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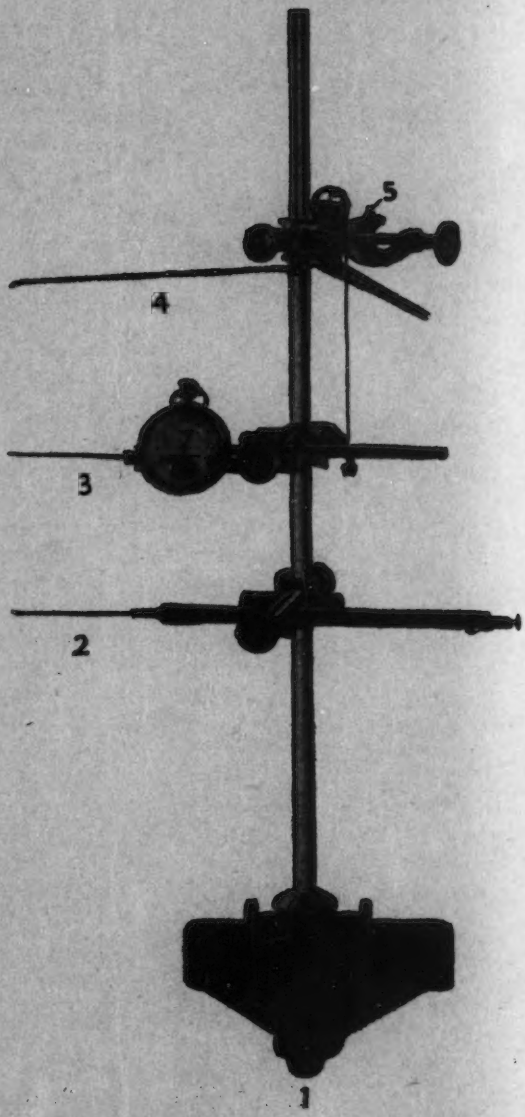

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SOME PHASES OF LIVER AND GALL BLADDER FUNCTION

Observations in Man on the Elimination of Methylene Blue

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Received for publication September 13, 1932

Recent observations on the elimination of methylene blue by the biliary system of rabbits and dogs furnished illuminating information regarding the functioning of the extrahepatic biliary passages and the gall bladder in these animals (1). Opportunities were sought, therefore, to determine whether similar studies in man would yield like data of value.

Method. In the *first group* of observations, methylene blue (Parke, Davis medicinal, one and two grain chocolate coated pills) was given by mouth, in doses of 10 mgm. per kilogram of body weight, to patients who drained bile either through a cholecystostomy or through a T-tube placed into the common bile duct following cholecystectomy. The bile was then collected for one day: in half hourly samples for 12 hours and in three-hourly samples for the remaining period.[1]

In the *second group* of observations, a single dose of methylene blue was similarly administered to patients: a, whose gall bladders had been removed two to three weeks previously; b, with cholecystgastrostomy; c, with cirrhosis of the liver, and to normal individuals. In all of these the urines were collected for 48 hours: in two-hourly samples for the first 12 hours; an eight-hourly night specimen; two-hourly samples during the second day; one eight- and then one two-hourly specimen.

In the *third group* of observations, methylene blue was administered in doses of 20 mgm. per kilogram of body weight, to one patient for 21 days and the urines collected in twelve hourly specimens.

Determinations of the methylene blue content of the bile and urine of each specimen were made by the quantitative method of Halpert and Hanke (2).

[1] A brief summary of the results of some of these observations was read (by title) at the Forty-eighth Session of the American Association of Anatomists (3).

Methylene blue in the bile. In the three patients—two of whom drained bile through a cholecystostomy and one through a T-tube placed into the ductus choledochus following cholecystectomy—methylene blue appeared in the bile by the third hour after the ingestion of the dye. The highest concentration—1:800 (Mr. A. R.), 1:7000 (Mr. F. R.) and 1:5600 (Mrs. B. K.) respectively—was reached between the fourth and seventh hours. A concentration of approximately 1:10,000 was maintained for 2 to 4 hours. The dye disappeared from the bile between the 12th and the 19th hours. The total amount of methylene blue recovered in the bile was: 1.09, 2.10 and 1.07 per cent respectively.

To a woman, aged 57 years, prior to operation, methylene blue was given by mouth in a dose of 20 mgm. per kilogram of body weight. One-half of this dose was given 86 and the other 74 hours before laparotomy. The patient was kept on the usual hospital diet. The dye disappeared from the urine 36 hours before the operation. At operation, 74 hours after the last dose of methylene blue, the bile in the gall bladder contained the dye in a concentration of 1:4800.

Methylene blue in the urine. In two of the above patients—one with the T-tube in the ductus choledochus (Mrs. B. K.) and one with cholecystostomy (Mr. A. R.)—methylene blue appeared in the urine 3 and 4 hours after ingestion, reaching its highest concentration—1:1300 and 1:6900—at the 8th and 10th hours. A concentration of approximately 1:8000 was maintained for 6 and 22 hours. The dye disappeared from the urine at the 32nd and 38th hours. The total amount of methylene blue recovered in the urine was 12.8 and 47.7 per cent respectively.

In the ten patients whose gall bladders had been removed 10 to 19 days previously, methylene blue appeared in the urine 2 to 6 hours following the ingestion of the dye and reached highest concentration—1:2400 to 1:26,600—between the 4th and the 22nd hours. The dye disappeared from the urine between the 30th and 48th hours. The total amount of methylene blue recovered in the urine varied between 7 and 39 per cent.

In the two patients in whom cholecystgastrostomies had been performed 14 and 19 days previously, methylene blue appeared in the urine about the 4th hour after ingestion of the dye. The highest concentration—1:1690 and 1:4500—was reached at the 6th and 12th hours respectively. The dye disappeared from the urine between the 38th and 48th hours. The total amount of methylene blue recovered in the urine was 17 and 18 per cent.

In the two patients with cirrhosis of the liver, methylene blue appeared in the urine about the 4th hour after ingestion of the dye, and reached highest concentration—1:1900 and 1:4500—at the 4th and 6th hours respectively. The dye disappeared from the urine at the 32nd and 38th hours. The total amount of methylene blue recovered in the urine was 12 and 19 per cent.

In the three normal individuals, methylene blue appeared in the urine between the 2nd and 4th hours following oral administration of the dye. It reached highest concentration—1:3200 to 1:4100—between the 4th and 6th hours, and disappeared from the urine at about the 46th hour. The total amount of methylene blue recovered in the urine varied from 14 to 35 per cent.

TABLE 1

Summary of data on the elimination of methylene blue in the urine following ingestion of 10 mgm. of methylene blue per kilogram of body weight: a, in patients with cholecystectomy; b, with cholecystgastrostomy; c, with cirrhosis of the liver, and d, in normal individuals

	PATIENT		FIRST APPEARANCE OF DYE IN URINE	HIGHEST CONCENTRATION OF DYE IN THE URINE		DURATION OF EXCRETION	TOTAL AMOUNT OF DYE RECOVERED
	Name	Age		Reached within	Dilution		
		years	*hours*	*hours*		*hours*	*per cent*
a	Mrs. J. C.	36	4	6	1:2,400		39
	Mrs. B. D.	50	4	10	1:3,000	36	29
	Mrs. S. G.	37	4	4	1:4,700	42	21
	Mrs. R. I.	33	2	6	1:5,000	48	8
	Mr. C. F.	43	2	6	1:6,000	30	13
	Mrs. L. P.	47	4	22	1:7,500	48	11
	Mrs. B. H.	38	4	8	1:14,000	38	7
	Miss A. G.	36	6	22	1:15,000	48	10
	Mrs. M. S.	64	2	4	1:17,000	48	15
	Mrs. M. B.	48	4	22	1:26,600	46	9
b	Mr. J. K.	50	4	6	1:1,690	48	18
	Mrs. R. L.	36	4	12	1:4,500	38	17
c	Mr. P. G.	52	4	4	1:1,900	32	19
	Mr. J. S.	56	4	6	1:4,500	38	12
d	Mr. J. K.	63	2	6	1:3,200	46	18
	Mr. D. M.	24	2	4	1:3,500	46	35
	Mr. N. A.	24	4	6	1:4,100	46	14

To Mr. J. K., aged 63 years, a known typhoid carrier, methylene blue was administered in daily doses of 20 mgm. per kilogram of body weight for 21 days. The concentration of methylene blue in 12-hour urine specimens varied between 1:6,000 and 1:1,660.

COMMENT. The data on the elimination of methylene blue in the bile of the human, following oral administration of the dye, are of particular interest. After oral administration of methylene blue to rabbits the dye was present in the gall bladder 42 hours subsequent to its disappearance from the hepatic bile. In the human, the dye appeared in the bile by the

3rd hour and disappeared between the 12th and 19th hours. In one instance the gall bladder bile contained methylene blue 72 hours after ingestion and 36 hours after the dye had disappeared from the urine.

In the human urine, methylene blue appeared between the 2nd and 6th hours following oral administration of the dye. It disappeared from the urine of the rabbit about the 36th hour, from that of the human between the 32nd and 48th hours.

These observations suggest close similarity in the mechanism of the elimination of methylene blue by the biliary and urinary systems of the human and the rabbit.

Substances reaching the liver may by selective action of the liver cells enter the bile or reach the general circulation by the efferent blood stream. Some of the ingested methylene blue is excreted in the bile, some is eliminated in the urine. It seemed plausible therefore to assume that damage to the liver cells would influence the passage of the dye in the one or the other direction, and thus the amount of methylene blue appearing in the urine might serve as an index of liver function. The variations in the highest concentrations, the periods in which these were reached, and the variations in the total amount of methylene blue recovered were, however, too great to permit any generalization (table 1).

SUMMARY AND CONCLUSIONS

Methylene blue appeared in the human bile by the third hour following its ingestion in doses of 10 mgm. per kilogram of body weight. The dye reached highest concentration—1:800 to 1:7000—between the fourth and seventh hours, and disappeared from the bile between the twelfth and nineteenth hours. The total amount of methylene blue recovered in the bile varied from 1.07 to 2.10 per cent of the amount administered.

In the urine, methylene blue appeared between the second and sixth hours, reached highest concentration—1:1690 to 1:26,600—between the fourth and twenty-second hours, and disappeared between the thirty-second and forty-eighth hours. The total amount of methylene blue recovered in the urine varied from 7 to 46.7 per cent.

The observations presented suggest close similarity in the mechanism of the elimination of methylene blue by the biliary and urinary systems of the human and the rabbit.

The variations in the elimination of methylene blue as to time, concentrations, and total amounts recovered could not be brought into any relation with disturbances of liver function.

Untoward effects were not noted during or after oral administration of methylene blue in daily doses of 20 mgm. per kilogram of body weight to an adult for a period of three weeks.

BIBLIOGRAPHY

(1) Rewbridge, A. G., M. T. Hanke and B. Halpert. This Journal, 1930, xcv, 511.
(2) Halpert, B. and M. T. Hanke. This Journal, 1929, lxxxviii, 351.
(3) Halpert, B., D. R. Mills and W. J. German. Anat. Rec., 1932, lii, 55.

THE CHEMICAL CONSTITUTION OF THE CONJUNCTIVA, CHOROID AND IRIS

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This paper is one of a series of articles on the chemical constitution of the eye (1) (2). There are practically no reports of investigations on the chemistry of the conjunctiva, choroid, or iris in literature. The chemical study performed on histological sections is not really applicable to the chemical study of the constitution of eye tissues as it is given in this report.

Unfortunately, the material for further investigation of the constituents of ocular tissue must be collected in small amounts, and be allowed to accumulate until a sufficient quantity is obtained for chemical analyses, the reports of which will follow in subsequent communications.

The constituents of the various tissues of the eye must be regarded as being in relative and not in absolute fixed amounts. The rapid progress of biochemistry in developing new methods and technique perhaps will show other and different relationships of the tissues. Nevertheless, the analysis of the constitution of the tissues as obtained in this study will be an aid in the study of ocular physiology and pathology.

MATERIALS. Fresh bovine eyes obtained about one hour after the death of the animals were dissected free from all extraneous matter. All abnormal or diseased eyes were discarded. Without the use of water, the conjunctivas were removed as rapidly as possible in a room saturated with water. Care was taken to remove only the thin conjunctiva and no extraneous tissue. The conjunctivas were placed in glass stoppered weighing bottles and dried at 105°C. to a constant weight. The corneas were then excised and the irises, without the ciliary body, were removed. The irises were lightly touched with filter paper to remove the free aqueous humor and then dried in the same manner as the conjunctivas. The lens, vitreous, and retina were rapidly taken from the eye and prepared for other analyses. The choroid was easily lifted off from the sclera and dried in the same way as the other two tissues. Since it was impossible to separate quantitatively the retinal pigment epithelium from the bovine choroid, as it is done in the human eye, the entire epithelial pigment was allowed to remain with the choroid. Five samples of about 2 grams each of conjunctiva, 8 samples of about 10 grams each of choroid, and 8 samples of about

8 grams each of iris were used. The percentages of solids in the tissues were as follows: conjunctiva, average 18.12, minimum 17.56, maximum 18.67;

TABLE 1

Protein and non-protein water soluble substances

TISSUE	AVERAGE PER CENT	MAXIMUM PER CENT	MINIMUM PER CENT
Conjunctiva			
Protein	13.66	16.85	10.36
Non-protein	8.32	10.54	6.59
Choroid			
Protein	13.66	18.14	9.28
Non-protein	8.32	10.63	7.13
Iris			
Protein	5.44	7.42	3.98
Non-protein	6.30	7.47	5.31

TABLE 2

Insoluble proteins

TISSUE	AVERAGE PER CENT	MAXIMUM PER CENT	MINIMUM PER CENT
Conjunctiva			
Mucoid	8.77	9.42	7.78
Collagen	61.93	64.05	60.23
Elastin	6.23	5.67	7.09
Choroid			
Mucoid	12.35	13.81	10.31
Collagen	27.93	29.25	26.33
Elastin	8.93	9.43	7.57
Pigment	19.01	20.05	18.16
Iris			
Mucoid	17.74	18.24	17.09
Collagen	38.62	40.21	37.13
Elastin	3.56	3.91	2.77
Pigment	19.05	20.60	17.89

TABLE 3

The chemical constitution of the conjunctiva, choroid, and iris

CONSTITUENT	CONJUNCTIVA		CHOROID		IRIS	
	Fresh	Dry	Fresh	Dry	Fresh	Dry
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Water	81.88		82.74		81.34	
Solids	18.12		17.26		18.66	
Inorganic matter	0.66	3.63	0.94	5.58	0.83	4.46
Organic matter	17.46	96.37	16.32	94.42	17.83	95.54
Insoluble proteins	13.93	76.94	8.51	49.21	11.18	99.92
Mucoid	1.59	8.77	2.13	12.35	3.31	17.74
Collagen (gelatin)	11.21	61.93	4.83	27.93	7.20	38.62
Elastin	1.13	6.23	1.55	8.93	0.67	3.56
Pigment			3.28	19.01	3.55	19.05
Albumin and globulin	1.84	10.13	2.37	13.66	1.02	5.44
Water-soluble extractives	1.25	6.89	1.43	8.32	1.18	6.30
Fat (ether-soluble matter)	0.44	2.41	0.73	4.22	0.90	4.83

choroid, average 17.26, minimum 16.54, maximum 17.92; iris, average 18.66, minimum 17.88, and maximum 19.31 (see table 1).

Inorganic matter (ash). The above samples were incinerated in platinum

crucibles in an electric furnace at about 500°C. to constant weight. The percentages of ash in the tissues were: conjunctiva, average 3.63, minimum 2.50, maximum 4.11; choroid, average 5.58, minimum 4.10, maximum 6.01; iris, average 4.46, minimum 4.07, and maximum 5.20 (see table 2). The average percentages of ash in tissues which were repeatedly extracted with water and dried, were as follows: conjunctiva 1.37, choroid 2.05, and iris 2.73.

Fat (ether-soluble lipids). The tissues were dried at 105°C. to constant weight. Samples of about 1 to 2 grams of conjunctiva and 5 to 10 grams of choroid and iris were extracted in a Soxhlet apparatus with anhydrous ether until lipids were no longer obtainable. The standard method of extraction was used in order to obtain comparable data. Seven samples of each tissue were used. The percentages of ether-soluble lipids in the tissues were: conjunctiva, average 2.41, minimum 2.12, maximum 3.19; choroid, average 4.22, minimum 3.57, maximum 4.99; iris, average 4.83, minimum 4.09, and maximum 5.36. The average percentages of ether-soluble lipids in tissues which were repeatedly extracted with water and dried, were as follows: conjunctiva 1.02, choroid 1.78, and iris 2.39 (see table 3).

Soluble proteins (albumin and globulin, and non-protein, water-soluble extractives). The orbital conjunctivas were quickly and carefully dissected from all extraneous tissue and finely sectioned. They were extracted repeatedly with small portions of distilled water at 15°C. until no soluble substances were found. The extract and insoluble part were dried at 105°C. to constant weight. The dried residue of the soluble portion was then repeatedly treated with water at 15°C. to remove the non-protein water-soluble material. The residue of coagulated proteins and water-soluble non-protein extractives was dried at 105°C. to constant weight. The water-soluble proteins from the conjunctiva, choroid, and iris, in a 5 per cent sodium chloride solution at a pH 5.8, coagulated at 53 to 55°, 61 to 64°, and 70 to 73°C. The greatest amount of coagulum occurred at 61 to 64°C. The water-soluble substances in the choroid with the retinal pigment epithelium were determined in the same manner. Eight 1 gram samples of conjunctiva and ten 8 gram samples of choroid and iris were used. The percentages of soluble substances are given in table 1.

Mucoid. The water-soluble proteins and extractives were removed from freshly prepared tissue. The tissue was repeatedly extracted by stirring at 4°C. with 10 cc. of half-saturated lime water per gram of tissue, for 24 hours until mucoid was no longer obtained (3). The suspension of tissue was removed by centrifugation. One per cent acetic acid was added to the solution until a maximum precipitation of the mucoid occurred upon standing. The mucoid, after it was removed from the solution, was redissolved in half-saturated lime water and reprecipitated with acetic acid.

It was then washed with cold distilled water until the wash water gave no test for calcium. It was extracted with alcohol and then ether, and dried in a vacuum over sulphuric acid to constant weight. Samples of about 1 to 2 grams of conjunctiva and 10 to 20 grams of choroid and iris were used.

Collagen (gelatin). The samples of tissues used for the determination of mucoid were washed with cold distilled water and then with 0.1 per cent acetic acid until no test for calcium was given by the wash water. The tissues were then washed repeatedly with carbon dioxide-free distilled water until the water of extraction indicated a pH about 6.7. The tissues were dried at 105°C. to constant weight. They were heated to 95°C. for 6 hours in distilled water and repeatedly extracted in a like manner until only a slight test was obtained for gelatin in the water of extraction. The insoluble residue was extracted with alcohol and then ether, and dried at 105°C. to constant weight. The solution of gelatin was concentrated by evaporation until it was viscous. After prolonged centrifugation to remove any fine suspension, the solution was slowly poured with rapid stirring into a large volume of 95 per cent cold alcohol. The fibrous gelatin was removed and redissolved in a small amount of hot water and reprecipitated with alcohol. It was washed with ether and dried at 105°C.

Elastin. The insoluble residue, remaining after the conversion of collagen to gelatin, was considered to be elastin in the conjunctiva, and elastin and pigment in the choroid and iris. The pigment obtained from the washing of the choroid and iris, and insoluble residues from the same tissues were treated with a buffered solution of pepsin at a pH 3 at 37°C. until no further digestion took place, and then treated with a buffered solution of pancreatin at a pH 8.0 at 37°C. until digestion was complete. The pigment which remained insoluble was washed repeatedly with distilled water until free of amino-nitrogen. The pigment was washed with alcohol and then ether, and dried at 105°C. to a constant weight. The percentages of mucoid, gelatin, elastin, and pigment in conjunctiva, choroid, and iris are given in table 2.

Discussion. The amount of water was difficult to determine accurately in small amounts of tissue which dried quickly, and which contained varying amounts of lymph and blood. These latter substances also influenced the determination of the relationship of the dry weights of the tissues to each other. No manipulation could overcome this difficulty.

The ash may not be considered as the actual mineral matter in the tissue. During incineration, the formation of carbonates, phosphates, and sulfates from the organic substances increased the ash content. This reaction occurred to a less degree in the ashing of the soluble fraction of the tissue.

The water-soluble extractives were obtained chiefly from the blood and lymph, especially in choroidal tissue.

The fat or the ether-soluble fraction was determined under usual stand-

ard conditions. No attempt was made to extract the fat after hydrolysis of the tissue, or with a series of solvents such as benzene, acetone, and chloroform, as these procedures would serve no useful purpose at the present time.

The chief interest of this paper is the insoluble proteins (see table 3). The mucoid of the iris and choroid was easily precipitated by acetic acid. Doubtless, very small amounts of nucleoprotein unavoidably contaminated the mucoid. The elastin was considered to be a protein unhydrolyzable by water. The insoluble black residue remaining after peptic and tryptic digestion was called pigment, as is indicated in table 3. It was obviously not pure. Repeated extractions of the pigment with cold one-tenth normal sodium hydroxide dissolved a black substance which was precipitated from solution by the addition of acetic acid, and also by salting out with ammonium sulfate. A dilute alkaline solution of the pigment showed a complete spectral absorption, except in the red. A very light brown residue which was insoluble in alkali remained after the extraction by alkali. This residue suggests that the matrix of the pigment granules may be a keratin.

The material was obtained through the courtesy of Mr. R. L. Fox.

BIBLIOGRAPHY

(1) KRAUSE, A. C. Amer. Journ. Ophthal., 1932, xv, 422.
(2) KRAUSE, A. C. Arch. Ophthal., 1932, vii, 598.
(3) BUERGER, L. AND L. B. GIES. This Journal, 1901–1902, vi, 218.

RESPONSE OF THE ISOLATED GALL BLADDER TO CHOLECYSTOKININ

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There has been some conflict in reports on the responses of the gall bladder to extracts of duodenal mucosa,—responses, that is, to preparations of secretin and cholecystokinin. Thus Mellanby (1926) stated that a trace of pure secretin, added to a bath of warm oxygenated Ringer's solution, would produce a marked contraction of a gall bladder suspended in the bath. The exact composition of the bath, the species of animal, and the acidity or alkalinity of the secretin preparation are not stated. Since the report by Ivy and Oldberg (1928) that contractions could be produced in the canine gall bladder in situ by intravenous injections of cholecystokinin, Halpert (1930) tried some of the preparation furnished by Doctor Ivy in experiments with the isolated gall bladder of the dog. He obtained negative results.

In the experiments we wish to report here, we used the isolated gall bladder of the guinea pig. This organ, though it is so thin as to be quite transparent, is remarkably active even after twenty-four hours in a refrigerator; when such specimens were set up in a glass-walled thermostat for the purpose of making records, their vigorous contractions could be directly observed as the tracings were made. The bath in which the isolated organ was immersed was of a composition recommended by Sollmann and Rademaekers (NaCl 0.9 per cent; KCl 0.042 per cent; $CaCl_2$ 0.012 per cent; $NaHCO_3$ 0.03 per cent; glucose 0.1 per cent). It has a pH of 7.8 and has the advantage that the glucose-free stock solution can be kept without depositing a precipitate and without consequent changes of composition. It was oxygenated by a stream of air bubbling through a 0.1 per cent solution of sodium bicarbonate. Since the bath was changed by an arrangement of siphons and a pre-heating device after each test, and since each test required only about ten minutes, this method of controlling the carbon dioxide tension proved quite satisfactory; there was no evidence of any changes in the pH of the bath during the course of an assay. The entire gall bladder was taken; the fundus was fastened by a small spring-clamp to a fixed support and the end from which the cystic duct arises

was tied by a thread to a writing-lever. Our records were therefore records of changes in length. We did not observe any important difference between full and nearly empty bladders, and did not find any advantage in washing out the bile. The assays were generally made by making records from two gall bladders simultaneously and in the same bath. Sometimes, however, simultaneous tracings were made from gall bladder and ileum of the same animal.

Using the gall bladder of the guinea pig under the above conditions, we regularly obtained contractions on adding solutions of cholecystokinin.

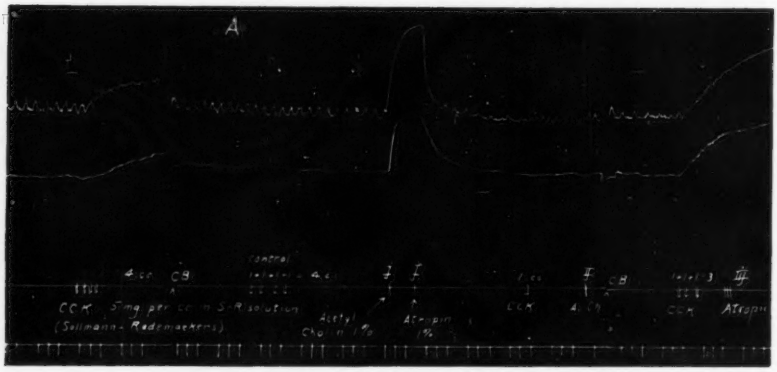

Fig. 1. Tracing made simultaneously from two isolated gall bladders of the guinea pig. The lower was fresh; the upper (more active) had been in the refrigerator 26 hours. Time-signals = 30 seconds; CCK = cholecystokinin; CB = change of bath; j = one drop. The CCK, in this instance dissolved in some bath-fluid with the aid of heat, caused contraction. Change of bath caused immediate relaxation. A sample of bath-fluid, heated and cooled to parallel the CCK solution, had no effect. Acetyl cholin caused a strong contraction. This was stopped by atropin, after which additional acetyl cholin had no effect. After a change of bath, CCK caused a slow, powerful contraction which was not stopped by three times the previous dose of atropin.

The contractions were slow, like those recorded by Ivy and Oldberg in the dog, and at the height of a contraction a marked rhythmic activity was likely to develop. These contractions were not abolished by atropin in doses which absolutely stopped the action of pilocarpin under the same conditions.

However, it was found that even the purest preparations of cholecystokonin were difficultly soluble in distilled water or in the fluid of the bath; and it was necessary to suspend the powdered material in water and dissolve it by acidifying with dilute HCl. It was then brought to the appropriate pH by the addition of dilute NaOH, using methyl red and phenol

red as indicators. Further controls were run to ascertain the possible effect of small changes in isotonicity and of temperature. We also tested cholecystokinin preparations inactivated by heat, in order to exclude the possibility of the protein in the preparation causing the contraction.

Further, since solutions of cholecystokinin are uniformly and characteristically foamy, a few other foamy materials were tried to see if surface tension was a factor. And, finally, the effect of varying the acidity of the preparations was studied. The results are summarized in the table.

From these results it is clear that the cholecystokinin was effective regardless of whether it was administered in acid or alkaline solution. The other materials tested all caused contractions in alkaline solution, but none in acid.

TABLE 1

Responses of isolated gall bladder (guinea pig)

pH	CHOLECYSTOKININ	CCK INACTIVATED BY HEAT AND ALKALI
5.0	++++−	−−
7.0	++−++++++?	−−
7.8	+++++++	−+
8.0	+−++++++++	
8.5	++?++++	++

pH	YOLK	HEATED YOLK	SERUM ALBUMEN	EGG ALBUMEN	Na TAURO-CHOLATE
5.0	−−−−?	−−−	−	−	−−
7.0	−−−+−−	−?	−	−	
7.8	−−+−−+−	++		−	
8.0					

In this table each sign (+, −, or ?) represents an assay and its result, whether positive, negative, or doubtful.

A relatively large dose of cholecystokinin was needed to produce these effects. The volume of the bath was 200 cc. Addition of one drop of 1 per cent acetyl cholin or 1 per cent pilocarpin caused an immediate strong contraction. With either drug present, the further addition of one drop of 1 per cent atropin caused immediate relaxation. After atropin, the gall bladder would not respond to pilocarpin or acetyl cholin, but it would respond vigorously to cholecystokinin or to histamin. The cholecystokinin solutions were made up to contain 5 mgm. per cc. From 3 to 10 cc. of such solutions were needed in the 200 cc. bath to evoke the contraction. There were present in the bath, therefore, about 50 times the total amount of cholecystokinin needed to produce a contraction in the gall bladder in situ in the barbitalized dog on intravenous injection.

The explanation of the necessity of this large dose may lie in the fact

that cholecystokinin is not diffusible. If this property hinders its action upon the tenuous gall bladder of the guinea pig, it may explain the lack of visible effect when cholecystokinin is applied to the relatively much thicker, whole gall bladder of the dog.

The responses noted above were also obtained in Tyrode's and in Locke's solution. Strips of ileum always behaved like the bladder as regarded response to cholecystokinin and to the drugs pilocarpin, acetyl cholin, atropin and histamin; the only difference noted was that the ileum did very poorly in Locke's solution.

SUMMARY

Earlier observations that extract of duodenal mucosa will cause contractions in the isolated gall bladder have been confirmed in the case of the guinea pig under conditions excluding possible effects of acidity and other disturbing factors.

A gall bladder made incapable, by atropinization, of responding to pilocarpin or acetyl cholin, can still respond to cholecystokinin.

BIBLIOGRAPHY

Halpert and Lewis. 1930. This Journal, xciii, 506.
Ivy and Oldberg. 1928. This Journal, lxxxiv, 599.
Mellanby. 1926. Lancet, ccxi, 215.

THE COMPARATIVE EFFECTS UPON METABOLISM OF INTRAVENOUSLY INJECTED TYROSINE, DIIODOTYROSINE, DIIODOTHYRONINE AND THYROXINE

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The origin of thyroxine from tyrosine is not as yet proved, but the chemical relationship between them suggests the advisability of a comparison between their metabolic effects. When given by mouth, tyrosine has been shown to have a specific dynamic action; the effect of thyroxine, however, is obviously much greater. In commenting upon this, Rapport and Beard (1927) stated, "In view of the possible relationship of the aromatic acids to the internal secretions of the thyroid and the adrenal medulla, the observed effects of phenylalanine and of tyrosine upon metabolism induce interesting speculations. If these two acids be indeed precursors of thyroxin and epinephrine, in what way, for example, is the stimulating potential of the former raised in the course of their elaboration to the hormones?"

We undertook the present work with the purpose of observing whether the addition of certain chemical radicals to the tyrosine molecule would quantitatively alter the effect upon metabolism, and of seeing whether this would throw light upon the chemical reasons for the effects of thyroxine. For example, can it be said that the thyroxine effect is due to the space-lattice arrangement of the molecule, or something else peculiar to itself, is it a quantitative summation of the individual effects of specific radicals, or is it a combination of different factors, as would seem most likely?

We have used, in addition to tyrosine and thyroxine, two substances which might lie in the metabolic pathway between them, namely, diiodotyrosine and diiodothyronine.[1] In the case of diiodothyronine, a phenyl group and a presumably inert oxygen atom are added to diiodotyrosine. In the cases of diiodotyrosine and thyroxine, two iodine atoms in the 3, 5 positions are added to tyrosine and diiodothyronine respectively. We

[1] At the friendly suggestion of Prof. Karl Thomas, of the Physiologisch-Chemisches Institut at the University of Leipzig, the Schering-Kahlbaum Chemische Fabriken of Berlin kindly gave us 10 grams of diiodothyronine, and we wish to acknowledge here our grateful thanks.

would have been glad to have at our disposal thyronine, bearing the same chemical relation to tyrosine as does diiodothyronine to diiodotyrosine, but so far have been unable to obtain enough of this material for our purposes.

EXPERIMENTAL METHODS. The experiments have been carried out on three adult female dogs living on a constant maintenance diet. The experimental materials were injected intravenously about 18 hours after the last feeding. Thyroxine (Hofmann-LaRoche) was injected up to 16.5 cc. of a 1/1000 solution, as bought. The pH of this solution was not determined. In the case of the other substances, their insolubility in water made it necessary to dissolve them in alkali. It was found that a 10 per cent solution of tyrosine in N/1 NaOH could be made, the buffering action of the tyrosine bringing the pH of the solution to 10.2[2], approximately equivalent to a N/10,000 NaOH. No more than 10 cc. of this solution was injected at any time, the injection being made during a period of several minutes. The buffering action of diiodotyrosine was also considerable, a 20 per cent solution in N/1 NaOH having a pH of 9.0. Not more than 2.5 cc. of this solution was injected. A 2 per cent solution of diiodothyronine in N/10 NaOH, of which not more than 7.25 cc. were injected, had a pH of 10.9. In all of these cases, we felt justified in neglecting the influence of these small amounts of alkali on the metabolism, particularly in view of the relatively prolonged effects of the materials upon the gaseous exchange. The solutions as used were freshly made, and except for tyrosine, not heated above the body temperature, as we observed, in the case of diiodotyrosine, a darkening of the solution and the odor of iodine on excessive heating or standing overnight.

After catheterization and the injection of the substances to be studied, the dog was put in the animal chamber of a closed system of the Benedict-Homans type and the gaseous exchange obtained as soon as practicable—the earliest period being between ½ to 1 hour after the injection—and thereafter until the metabolism had returned to normal, or as long as the animal would remain quiet, in those experiments where the rise in heat production was prolonged. In these latter experiments, points were established on succeeding days by obtaining the gaseous exchange for a period long enough to make certain of the actual height of metabolism. Where, as in the case of tyrosine or diiodotyrosine, the increased metabolism persisted for less than 24 hours, the later hours of the rise would be obtained in another experiment. Thus, in experiment 18 on dog 19 only the first 5 hours after tyrosine could be obtained, hence on a later date, the dog was put in the box 4 hours after the injection, and the experiment con-

[2] We are indebted to Dr. M. G. Banus for the electrometric pH determinations of the solutions used.

tinued until the metabolism had returned to normal, which in this case was 6 hours later.

The basal metabolism in each of the three dogs we used did not deviate more than a maximum of ±0.5 Cal. per hour. (In dog 18 the maximum was 0.2 Cal.)

During the period of experimentation 20 alcohol checks were carried out, the average respiratory quotient being 0.660, an error of slightly more than 1 per cent; the maximum deviation from the theoretical of 0.667 being 0.654, an error of about 2 per cent.

EXPERIMENTAL RESULTS. A. *Tyrosine.* Lusk (1912) found that the oral administration of 20 grams of tyrosine to a dog of about 10 kilos raised the metabolism 13.5 per cent. Rapport and Beard (1927) obtained rises

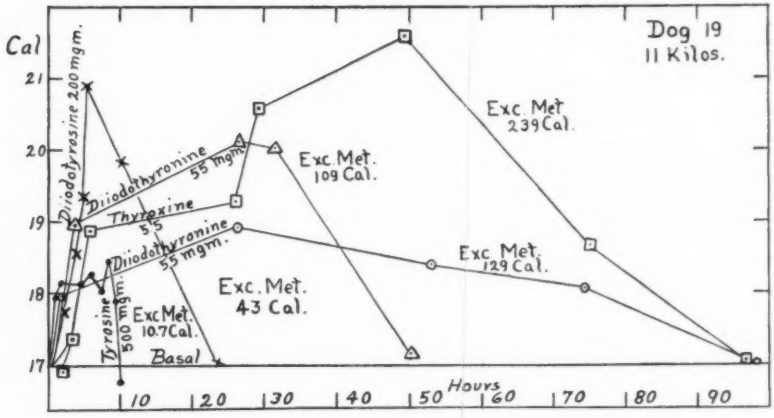


Fig. 1

of 21 and 16 per cent on giving 10 grams of the amino acid to a somewhat smaller dog. In view of the fact that in neither of these cases was the urinary nitrogen appreciably elevated, and in view also of the failure of Folin and Denis (1912) to find increase in the urea and non-protein blood nitrogen after injecting tyrosine into a loop of gut, we were inclined to the view that only a small amount of tyrosine was being absorbed into the blood after oral administration, and that upon injection of relatively large amounts directly into the blood stream, a large specific dynamic action would be observed. However, 200 mgm. dissolved in N/1 NaOH produced no change in the total metabolism. In dog 19, 500 mgm. raised the heat production a total of 10.7 Cals., the effect lasting 10 hours. In dog 20 this amount produced no appreciable result, while 1.0 gram pro-

duced a total rise of 15 Cals. In dog 18, no satisfactory tyrosine experiment was obtained (figs. 1 and 2). These surprisingly small effects on the heat production after intravenous injection make us question our original hypothesis of slow or incomplete absorption of the amino acid from the gastro-intestinal tract. An explanation of the results may possibly be found in the observation of King and Rapport (1933) that after intravenous injection tyrosine disappears almost at once from the blood, and that there is no enhanced urea formation. It can probably still be said that per gram metabolized, the specific dynamic action is large compared with most of the other amino acids.

B. *Diiodotyrosine.* The effect of diiodotyrosine was appreciably greater than that of tyrosine (figs. 1, 2, and 3). Whereas in dog 19 0.5 gram of

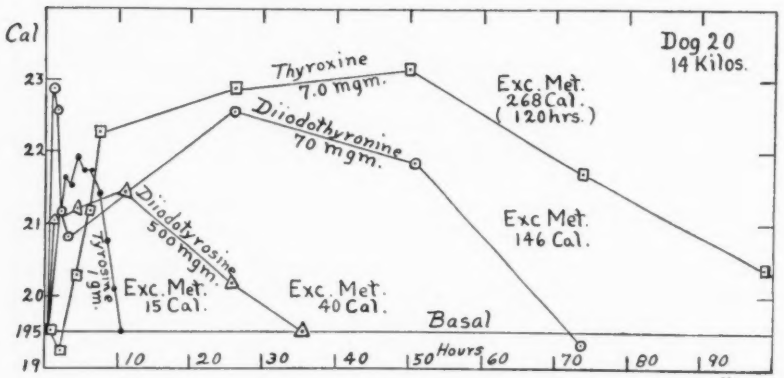


Fig. 2

tyrosine elevated the metabolism only 10.7 Cals., 200 mgm. of diiodotyrosine resulted in an increase of 43 Cals. In dog 20, the injection of 500 mgm. of diiodotyrosine raised the metabolism 40 Cals., the higher level lasting 30 hours. In the two dogs the ratio to the tyrosine effect was not constant, being 10 to 1 in one case and 5.5 to 1 in the other. In dog 18, a total increase of 69 Cals. above the basal level followed the injection of 500 mgm. of diiodotyrosine.

C. *Diiodothyronine.* In the case of this substance, the response to injection was not only greater, but of longer duration, than that of both tyrosine and diiodotyrosine (figs. 1, 2 and 3). In dog 19, two experiments were performed, 55 mgm. in 3 cc. N/10 NaOH being injected in each instance (0.5 mgm. per kilo). In one experiment the metabolism was elevated about 50 hours, the total excess being 109 Cals.; in the other, the increase was 129 Cals., or almost the same as before, although the height-

ened metabolism lasted almost twice as long. In dog 20, 70 mgm. of the material (0.5 mgm. per kilo) caused a rise of 146 Cals., lasting about 72 hours. The response to an injection of 0.5 mgm. per kilo (55 mgm.) in dog 18 was relatively less than in the other two dogs, amounting to only 61 Cals., the effect lasting less than 48 hours. Tripling the amount injected caused a rise of 284 Cals., lasting almost 100 hours, a more than proportional increase in response.

D. *Thyroxine.* In dog 18, the response to thyroxine was also less than in the other two dogs. The injection of 0.5 mgm. per kilo (5.5 mgm.) caused the metabolism to rise only 46 Cals.; with 16.5 mgm., however, an increase of 302 Cals. was obtained, the effect lasting about 100 hours. In dog 19, the administration 0.5 mgm. per kilo resulted in an excess of

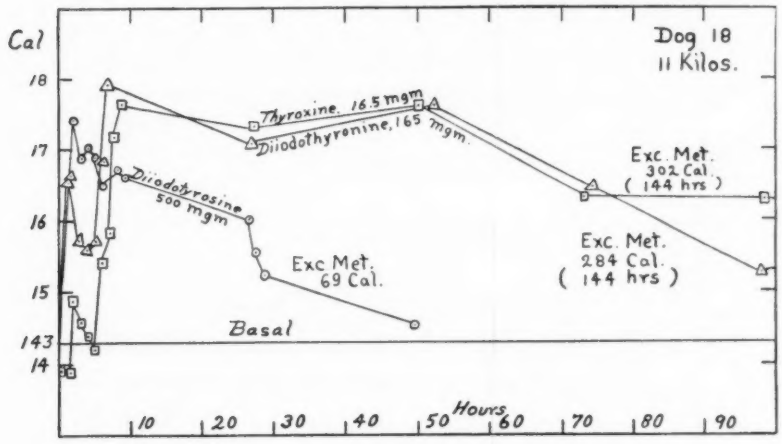


Fig. 3

239 Cals., lasting 100 hours; and in dog 20 the response was even greater to a similar dose per kilo of weight—an excess 268 Cals., lasting 120 hours.

E. *The initial hours.* In figure 4 will be seen the response of the various substance in each dog during the early hours after the injection of the material. This is of interest chiefly because thyroxine differs markedly from the other three substances in the rapidity with which the effect of the injection becomes manifest. Tyrosine and diiodotyrosine rise to a maximum, or nearly so, in the earliest observations, maintaining an irregular plateau for a varying time thereafter before returning to the basal level. (An exception is seen in dog 19, where the maximum for diiodotyrosine was not reached until the 5th hour, the response in the first two hours being much smaller than in the other dogs.) Diiodothyronine also pro-

duces a quick response, though the maximal effect is usually not observed until 24 hours later. In the case of thyroxine, however, there is either no effect, or only a slight and somewhat questionable one, during the first 4

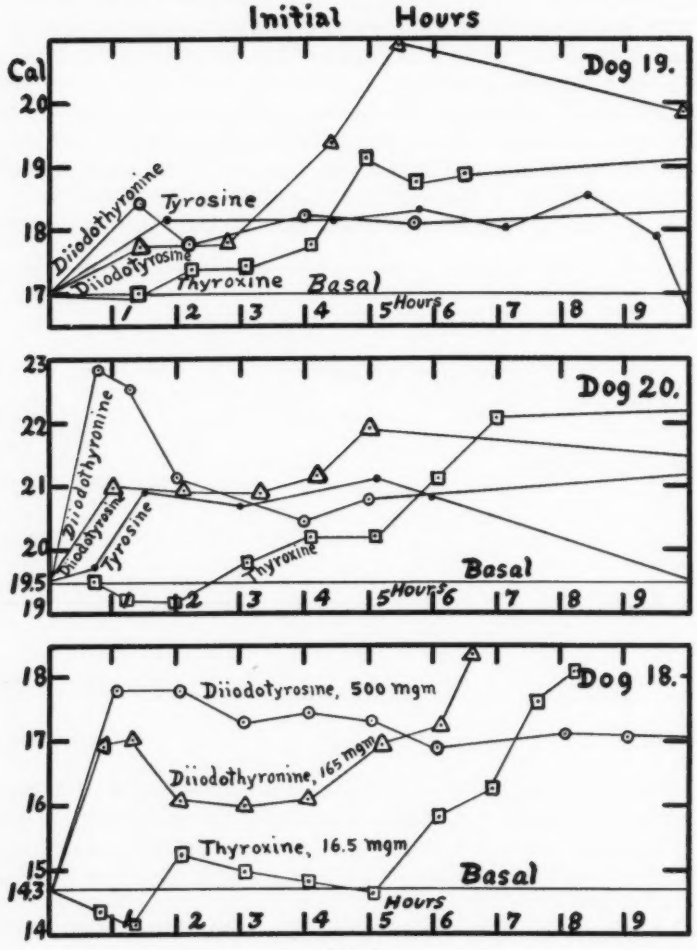


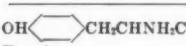
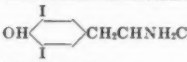
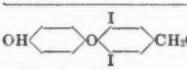
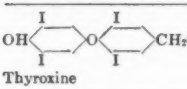
Fig. 4

to 5 hours after injection, after which the rate of metabolism rises steeply, the maximum effect being delayed until succeeding days.

F. *Urinary N.* After tyrosine the average nitrogen excretion of the 3 dogs rose to 0.173 gram per hour during the period of elevated metabolism,

from a post-absorptive level of 0.144, returning to 0.147 the next day. The rise did not always occur. After diiodotyrosine there was, during the first 8 to 10 hours a fall in the nitrogen, the average being 0.117 gram per hour, the average basal level before these experiments being 0.132. On the day after the metabolism had returned to normal the excretion averaged 0.148 gram per hour. Diiodothyronine had practically no effect on the nitrogen, the average basal value before and after being 0.139 and 0.136 gram per hour, respectively, while on the days of increased metabolism the average was 0.129 gram per hour. In the case of thyroxine, the average level before injection was 0.122 gram per hour, which remained practi-

TABLE 1

	INCREASE IN METABOLISM PER GRAM PER KILO AND RATIO OF EFFECT									
	Dog 19		Dog 20		Dog 18		Average			
	Cals.	Ratio	Cals.	Ratio	Cals.	Ratio	Cals.	Ratio		
 Tyrosine	235	1	210	1			223	1		
 Diiodotyrosine	2,365	10	1,109	5	1,513	1	1,662	7.5	1	
 Diiodothyronine	21,500				12,280	8				
	25,800	100	29,200	140	18,930	13	23,977*	110	15	1
 Thyroxine					92,000	60				
	478,000	2000	536,000	2500	201,000	130	405,000*	18,000	240	17

* Excluding the lower of the figures for dog 18.

cally the same on the day of injection; on the two succeeding days there was, on the average, a slight rise, to 0.148 and 0.149 gram per hour, respectively, thereafter returning to about the original. In 2 of the 5 thyroxine experiments, there was at no time an appreciable change in the urinary nitrogen.

On analysis, there appears to be no correlation between such slight changes in the nitrogen excretion as occurred and the height of metabolism.

G. *General comparison.* A quantitatively exact comparison is clearly impossible with the data at our disposal. Even with uniform reaction to any given substance, the same amounts of each could not be given on account of their widely varying influences upon metabolism, so that the reduction of their effects to that of 1 gram of the material per kilo of dog

has obvious defects. It shows in a rough way, however, the potency of the four substances, which is summarized in table 1. It will be seen on consulting this table that while the dogs do not react in quantitatively the same way to the various materials, there is in each case a similar slope of increased effect with the different substances, tyrosine being least effective, then diiodotyrosine, diiodothyronine and thyroxine in that order. Averaging the results, diiodotyrosine would appear to have 5 to 10 times as much effect as tyrosine on metabolism, two atoms of iodine in the 3, 5 position having been added to the molecule. When two atoms of iodine, however, are added in the same position to one of the phenyl groups of diiodothyronine, forming thyroxine, the increase in effect is greater, averaging 17 fold. On the formation of diiodothyronine from diiodotyrosine, involving the addition of another phenyl group, the influence on the energy exchange is increased 15 times. There emerges from this comparison no clear picture of the influence of added chemical radicals, and we are still in the dark as to whether it is their position in the molecule or some other factor which enhances the effect of the molecule as a whole. It would appear that iodine, as such, is not responsible, partly because its effect differs depending on the substance to which it is added, and partly because there is evidence indicating that inorganic iodine has no appreciable effect on the heat production of the normal animal.

This much is clear, however, that although thyroxine is in the neighborhood of 2000 times as powerful as tyrosine, the relation between the two, even from their effects on the total metabolism, cannot be neglected; for our data can be construed as a gradual progression of effect from tyrosine through substances that may well be intermediaries to thyroxine itself. Such is the chief point we would make.

SUMMARY

Tyrosine, diiodotyrosine, diiodothyronine and thyroxine were administered to dogs by intravenous injection, and the effects on the gaseous exchange studied. The maximum rise due to 1.0 gram of tyrosine was 10 per cent of the total metabolism, the effect lasting about 10 hours, the average total increase being about 220 Cals. per gram of tyrosine per kilo of animal. Diiodotyrosine produced about 7.5 times as much effect as did tyrosine; diiodothyronine about 15 times that of diiodotyrosine; and thyroxine about 17 times that of diiodothyronine. Generally speaking, the greater the total increase, the more prolonged the effect. The effect of all of the substances but thyroxine began almost at once (not later than ½ to 1 hour after administration); in the case of thyroxine an appreciable effect was delayed 4 to 5 hours.

Though the ratio of the effects of thyroxine and tyrosine is about 2000 to 1, our data would indicate a step-like progression in stimulating influ-

ence as iodine and the phenyl radical are added to the tyrosine molecule in certain positions. The significance of this is not at present clear.

BIBLIOGRAPHY

FOLIN, O. AND W. DENIS. 1912. Journ. Biol. Chem., xii, 141.
LUSK, G. 1912. Journ. Biol. Chem., xiii, 155.
RAPPORT, D. AND H. H. BEARD. 1927. Journ. Biol. Chem., lxxiii, 299.

THE FATE OF INTRAVENOUSLY INJECTED TYROSINE

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Tyrosine, when taken by mouth, has a comparatively large specific dynamic action. Lusk (1912) found that 20 grams of this amino acid, given to a dog weighing 9.7 kilos, increased the metabolism 13.5 per cent. Rapport and Beard (1927) obtained even larger effects, 10 grams of tyrosine provoking increases in two experiments of 21 and 16 per cent respectively. Yet Lusk found no increase in urinary nitrogen that he could not ascribe to possible experimental error, and in Rapport and Beard's experiments the rise in nitrogen elimination was also negligible. Moreover Folin and Denis (1912), upon injecting tyrosine into the intestine of cats, could find no increase of the non-protein and urea nitrogen of the blood, and only by a faint color with the phosphotungstic-phosphomolybdic acid reagent were they able to demonstrate any evidence of tyrosine in the blood. From the above results it appeared that tyrosine was absorbed with the blood only in small amounts from the intestine, and that per gram of the substance actually present in the blood, its effect upon metabolism must be enormous.

Acting on this supposition Canzanelli and Rapport have recently (1933) injected as much as 1.0 gram of tyrosine directly into the blood stream, an amount which, judging from the above-mentioned work, would seem to have been far more than could at any time be absorbed into the blood stream after the administration of 10 grams by mouth. It was expected by these investigators that there would under these circumstances be a large specific dynamic action. Yet actually the rise in metabolism was at no time greater than about 10 per cent, lasting about 10 hours. These findings made us wonder whether the failure of Folin and Denis to find evidence of significant increase of blood tyrosine, and of the other experimenters to observe an increase in urinary nitrogen, was in fact due to failure of absorption from the gastro-intestinal tract. We have therefore investigated the fate of tyrosine when injected intravenously.

EXPERIMENTAL METHODS. The work was carried out on dogs who were living on a constant maintenance diet of cracker meal, lard and lean beef heart, the ratio of carbohydrate, fat and protein being about 65, 15 and 20 per cent respectively of the total calories. The animals were starved for

36 hours before the tyrosine injection, which was made into the external jugular vein. On account of its insolubility, and the desirability of using a small volume of liquid, a solution was made with N/1 NaOH, the buffering action of the tyrosine bringing the pH of the injected solution down to 10.2,[1] or about the equivalent of a 1/10,000 N NaOH, which it was deemed safe to use. The substance was injected slowly, not faster than 50 cc. in 10 minutes, and no untoward effects were observed.

As soon as possible following the injection, at varying intervals thereafter for several hours, and at the end of 24 hours, samples of jugular vein blood were taken. In these samples NH_2 nitrogen, phenols, and in some cases urea nitrogen, were determined. Control analyses were made on blood samples taken before the injection, and after the return of the blood constituents to normal values. The urine was collected for 24 hours before and after the injection, and sometimes for a second 24 hour period on the day following, and analyzed for NH_2 nitrogen, total nitrogen, and phenols.

Blood and urinary NH_2 nitrogen were determined by the methods of Folin (1922), blood urea by the method of Folin and Wu (1919); urinary total nitrogen by the macro-Kjeldahl. Blood and urinary phenols were analyzed by a modification of the method of Folin and Marenzi (1929) for the analysis of tyrosine in proteins. For blood, this modification was as follows: 20 cc. of the Folin-Wu tungstate blood filtrate were used instead of 20 cc. of protein hydrolysate; the solution from the $HgSO_4$ precipitation was decanted after centrifuging into a 75 cc. Pyrex ignition tube instead of a 100 cc. volumetric flask; the final volume was 50 cc. instead of 100 cc. For urine, the same procedure was followed, except that 10 cc. of urine were used, and 10 cc. of water added before adding the 15 per cent $HgSO_4$ solution.

EXPERIMENTAL RESULTS. I. *Normal dogs.* a. *Blood phenols.* The method was checked by adding known quantities of tyrosine to blood as follows: 6.3 mgm. were added and 5.4 mgm. recovered; 0.5 mgm. added 0.49 mgm. recovered; 1.00 mgm. added and 1.08 recovered; 2.00 mgm. added and 1.92 recovered. As little as 0.05 mgm. gave an appreciable color in 50 cc. of solution. The method used cannot, naturally, be regarded as specific for tyrosine. However, the control blood did not show the presence of phenols by this technique, and we therefore felt justified in assuming that such quantities as we obtained after injecting tyrosine were, if not the amino acid itself, at least the products of its metabolism. With this qualification we have considered the results as one of the indices of the amount of tyrosine actually present in the blood.

Upon injecting 1 gram of tyrosine into normal dogs, no trace of tyrosine was found in the blood thereafter, even within 5 minutes after the injection

[1] We are indebted to Dr. M. G. Banus for the electrometric determination of the pH.

TABLE 1

Intravenous tyrosine injection in normal dogs

DOG NUMBER AND WEIGHT	EXPERIMENT NUMBER	TIME	BLOOD			URINE			REMARKS
			NH_2 nitrogen	Phenols	Urea	NH_2 nitrogen	Phenols	Total nitrogen	
			mgm. per 100 cc.			*mgm.*	*mgm.*	*grams*	
♯23	1	Tue. 4:30 p.m.				30.8*	8.0*	2.36*	Wed., July 7, 1931, 10:35
10.5 Kilos		Wed. 10:30 a.m.	5.0	none					to 10:40 a.m. 1 gm.
		1:15 p.m.				3.7	13.5	0.50	Tyrosine in 8 cc. N/1
		2:15 p.m.	5.6	none					NaOH
		3 p.m.				2.9	12.1	0.61	
		4:30 p.m.	5.0	none					
		5 p.m.				2.6	13.0	0.29	
		7:30 p.m.	4.9	none					
		8:30 p.m.				2.3	20.1	0.53	
		Thu. 10:30 a.m.	5.0	none		4.1	12.8	0.92	
						15.6*	71.5*	2.84*	
♯23	3	Tue. 4:30 p.m.				13.6*	35.9*	2.53*	Wed., July 29, 1931, 9:05
11.3 Kilos		Wed. 9:20 a.m.	6.5	none					to 9:15 a.m. 1 gm.
		12:20 p.m.	4.5	none		1.4	8.0	0.26	Tyrosine in 8 cc. N/1
		2:30 p.m.				1.1	6.7	0.22	NaOH
		5 p.m.	5.1	none		1.2	6.4	0.18	
		Thu. 9 a.m.	4.4	none		6.3	13.2	1.00	
						10.0*	34.2*	1.65*	
♯23	4	Tue. 4:30 p.m.				8.4*	39.5	2.39	Wed., Aug. 7, 1931, 9:55
11.1 Kilos		Wed. 10:10 a.m.	9.2	none					to 10:05 a.m. 1 gm.
		1:10 p.m.	5.7	none		1.4	15.0	0.24	Tyrosine in 8 cc. N/1
		5 p.m.				1.3 6.8*	11.6 36.3*	0.29 1.44*	NaOH
		Thu. 10 a.m.	5.1	none		4.1	9.7	0.91	
		Fri. 10 a.m.	5.7	none		6.4*	24.1*	1.89*	

#23 11.6 Kilos	7	Tue. 10:55 a.m.	4.8	7.0					Tues., Aug. 8, 1931, 10:40 to 10:50 a.m. 5 gm. Tyrosine in 45 cc. N/1 NaOH
		11:55 a.m.	4.1	none					
		2:25 p.m.	3.8	none					
		Wed. 10:55 a.m.	4.3	none					
#23 10.7 Kilos	10	Thu. 9:25 a.m.				15.6*	19.7*	2.25*	Thur., Aug. 22, 1931, 9:35 to 9:42 a.m. 5 gm. Tyrosine in 4 2cc. N/1 NaOH
		9:30 a.m.	4.1	none	7.6				
		9:45 a.m.	5.7	33.0	7.9				
		10:45 a.m.	4.1	none	7.6				
		1:50 p.m.	3.7	none	6.1				
		Fri. 9:29 a.m.				42.8*	53.6*	1.80*	
		9:25 a.m.	4.6	none	8.1				
		Sat. 9:20 a.m.				13.4*	15.5*	1.46*	
#20 13.8 Kilos	13	Thu. 9:30 a.m.				18.8*	59.1*	2.53*	Thur., Nov. 19, 1931, 9:40 to 9:55 a.m. 5 gm. Tyrosine in 50 cc. N/1 NaOH
		10 a.m.	7.7	9.0	9.9				
		1 p.m.	5.5	none	9.3				
		4 p.m.	5.5	none	8.1				
		Fri. 9:30 a.m.				28.8*	79.1*	3.05*	
		9:45 a.m.	6.1	none	6.2				
		Sat. 9:30 a.m.				17.9*	20.7*	2.16*	
#24 8.5 Kilos	15	Tue. 10:30 a.m.				19.2*	50.4*	1.44*	Tues., Dec. 22, 1931, 10:45 to 10:55 a.m. 5 gm. Tyrosine in 50 cc. N/1 NaOH
		10:40 a.m.	6.1	none	9.3				
		11 a.m.	11.2	23.4	7.9				
		1 p.m.	8.1	none	7.1				
		4 p.m.	6.9	none	6.9				
		4:30 p.m.				6.2 20.3 } 26.5*	214.5 62.1 } 276.6*	0.66 1.28 } 1.94*	
		Wed. 10:30 a.m.							
		10:40 a.m.	6.5	none	8.9				
		Thu. 10:30 a.m.				18.9*	31.7*	1.32*	

* Total 24 hour amount.

(table 1). In four experiments 5 grams of the amino acid were injected. Assuming the blood volume to be 7 per cent of the total weight of the animal, it can be calculated that in experiments 7, 10, 13 and 15, 610 mgm., 670 mgm., 520 mgm., and 840 mgm. of tyrosine respectively might have been present in each 100 cc. of blood after the injection. Actually, in the first sample, taken not more than 5 minutes following injection, 7.0, 33.0, 9.0, and 23.4 mgm., respectively, of phenol expressed in terms of tyrosine were found, indicating that 95 to 98 per cent of the tyrosine was no longer present in the blood stream. In later samples even this small quantity could not be found.

b. *Blood NH_2 nitrogen.* As will be seen in table 1, there was only a very small elevation in blood NH_2 nitrogen, averaging in 3 experiments only 2.5 mgm. per 100 cc. in the first sample after injection, and returning to normal in all later samples. After 5 grams of tyrosine the rise was still inconspicuous in experiments 7, 10 and 13, averaging 1.3 mgm. per 100 cc., and having returned to normal in two of these experiments in a sample taken 1 hour after the injection. In experiment 15 the rise was somewhat greater (4.7 mgm. per cent) in the first sample and was still definitely elevated 2 hours later. If these results be compared with those observed after injecting glycine (see table 2), it will be seen that even allowing for the greater nitrogen content of glycine the elevation of blood NH_2 nitrogen after giving the latter was relatively greater than after tyrosine. In none of the tyrosine experiments was more than a negligible amount of the nitrogen injected to be found in the blood.

c. *Blood urea nitrogen.* That this apparent disappearance of tyrosine from the blood was not associated with its breakdown via deaminization is shown by the blood urea figures in table 1. No appreciable increase in urea formation was apparent in the later hours, which are presumably the significant ones (cf. glycine, and see Van Slyke and Meyer, 1913). Recently Kiech and Luck (1932) have found that after injection of a suspension of tyrosine subcutaneously in rats, there is no subsequent increase in the total urea content of the animals, and attribute this to failure of absorption. Our present results would seem to indicate that this is not necessarily the explanation.

d. *Urinary findings.* The NH_2 nitrogen of the experimental day showed no change from the control 24 hours before injection when 1 gram of tyrosine was given; when 5 grams were administered, the increase in the urine was negligible, amounting at most (expt. 10) to 7 per cent of the material given. The phenols also showed no indication of increase, except in experiment 15, when 5 grams of tyrosine were injected, yet even in this experiment the rise could not account for more than 5 per cent of the amino acid. These findings, showing that the tyrosine had not been excreted in the urine to any extent, either as such, or as other phenols, together

TABLE 2

Intravenous glycine in normal and thyroidectomized dogs

DOG NUMBER AND WEIGHT	EXPERIMENT NUMBER	TIME	BLOOD		URINE			REMARKS
			NH_2 nitrogen	Urea	NH_2 nitrogen	Phenols	Total nitrogen	
Normal								
			mgm. per 100 cc.		*mgm.*	*mgm.*	*grams*	
#23	9	Thu. 10:34 a.m.	19.9					Thurs., Sept. 9, 1931, 10:25 to 10:32 a.m. 5 gm. Glycine in 28 cc. H_2O
11.6 Kilos		11:34 a.m.	6.3					
		2:30 p.m.	3.3					
		Fri. 10:30 a.m.			46.8*		3.14*	
		10:35 a.m.	4.2					
#24	14	Thu. 9:50 a.m.	5.6	10.9	16.7*	33.7*	1.47*	Thurs., Dec. 10, 1931, 9:50 to 9:55 a.m. 5 gm. Glycine in 30 cc. H_2O
9.1 Kilos		9:58 a.m.	20.7	9.9				
		10:50 a.m.	11.5	17.4				
		2 p.m.	6.4	13.4				
		4:30 p.m.	5.4	7.8	10.8 / 44.3 } 55.1*	12.1 / 14.0 } 26.1*	0.37 / 1.23 } 1.60*	
		Fri. 9:30 a.m.	5.3	8.3				
		Sat. 9:50 a.m.			18.9*	25.7*	1.99*	
Thyroidectomized								
#16	6	Thu. 10 a.m.	3.0					Thurs., Sept. 9, 1931, 10:05 a.m. 1 gm. Glycine
10.2 Kilos		10:15 a.m.	8.1					
		12:45 a.m.	3.1					

* Total 24 hour amount.

TABLE 3

Intravenous tyrosine injection in thyroidectomized dogs

DOG NUMBER AND WEIGHT	EXPERIMENT NUMBER	TIME	BLOOD			URINE			REMARKS
			NH_2 nitrogen	Phenols	Urea	NH_2 nitrogen	Phenols	Total nitrogen	
			mgm. per 100 cc.			*mgm.*	*mgm.*	*grams*	
#22	2	Tue. 4:30 p.m.				37.2*	23.9*	2.11*	Wed., July 15, 1931, 11:00 to 11:10 a.m. 1 gm. Tyrosine in 8 cc. N/1 NaOH
9.0 Kilos		Wed. 11 a.m.	4.7	none					
		1:15 p.m.				3.7	3.5	0.26	
		2:40 p.m.	6.5	none					
		3:15 p.m.				2.3	3.4	0.32	
		4:45 p.m.	4.7	none					
		5:15 p.m.				1.2	2.2	0.15	
		7:45 p.m.	4.5	none					
		8:45 p.m.				1.5	3.8	0.25	
		Thu. 11 a.m.	4.6	none		2.9	5.9	0.66	
						11.7*	18.8*	1.64*	
#16	5	Fri. 9:30 a.m.				6.1*	18.1*	2.52*	Fri., Aug. 28, 1931, 9:40 to 9:50 a.m. 1 gm. Tyrosine in 8 cc. N/1 NaOH
11.0 Kilos		9:50 a.m.	4.2	none					
		2:30 p.m.	4.2	none		1.82	8.0	0.67	
		5 p.m.				1.11 } 7.5*	4.0 } 27.9*	0.36 } 2.22*	
		Sat. 9:30 a.m.	5.7	none		4.59	15.9	1.19	
#15	11	Thu. 9 a.m.	7.5	none	9.9	11.7*	9.3*	2.61*	Thurs. Nov. 6, 1931, 9:05 to 9:20 a.m. 2.5 gm. Tyrosine in 25 cc. N/1 NaOH
7.1 Kilos		9:25 a.m.	9.3	none	10.3				
		10:25 a.m.	7.6	none	11.5				
		11:25 a.m.	6.9	none	8.6				
		Fri. 9:10 a.m.				26.8*	42.2*	2.37*	
		9:15 a.m.	6.9	none	7.8				

#22	8	Tue. 10:40 a.m.				18.2*	21.0*	2.87*	Tues., Sept. 16, 1931, 10:45 to 10:55 a.m. 5 gm. Tyrosine in 45 cc. N/1 NaOH
9.0 Kilos		11 a.m.	5.2	33.0					
		12 a.m.	3.6	none					
		2:30 p.m.	3.4	none					
		Wed. 10:40 a.m.				17.7*	155.0*	2.26*	
		11 a.m.	3.5	none					
		Thu. 10:40 a.m.				5.8*	6.3*	1.78*	
#17	12	Thu. 10 a.m.				20.2*	63.8*	2.12*	Thurs., Nov. 12, 1931, 10:10 to 10:25 a.m. 5 gm. Tyrosine in 45 cc. N/1 NaOH
6.9 Kilos		10:05 a.m.	6.5	none	5.9				
		10:30 a.m.	9.9	24.5	5.6				
		12:30 p.m.	6.5	none	5.7				
		3 p.m.	6.6	none	5.7				
		4:30 p.m.				10.7 / 16.4 } 27.1*	84.7 / 57.3 } 142.0*	0.61 / 1.88 } 2.49*	
		Fri. 10 a.m.							
		Sat. 10 a.m.				23.5*	49.1*	2.25*	

* Total 24 hour amount.

with lack of deaminization shown above, render such changes as occurred in the total nitrogen without significance. In three experiments the total nitrogen increased; in three there was a decrease, probably attributable to the continuance of starvation.

Shambaugh, Lewis and Tourtellotte (1931) have given tyrosine to rabbits by mouth, and using the method of Theis and Benedict, have observed an increase in free phenols in the blood. Their figures do not show an amount of blood phenol at all comparable with the amount introduced, and there was no appreciable increase in either the blood amino nitrogen or urea nitrogen for periods up to 30 hours after the administration of the material. Only 5 per cent of the ingested tyrosine was excreted as phenol. Our results with intravenous injections, which are very similar to these, indicate that the absence of evidence for the presence of tyrosine in the blood or for its metabolic breakdown in significant amounts when ingested by mouth, is not due to slow or incomplete absorption from the intestine or tissue spaces, but to its disappearance from the blood after arriving there.

II. *Thyroidectomized dogs.* On the basis of the above findings it seemed probable that tyrosine was being deposited in tissues somewhat after the manner of other amino acids as suggested by Van Slyke and Meyer, either in free form or in combination.

Marine and Rogoff (1917) observed that when KI was injected intravenously into dogs, there was within 5 minutes a large increase in the iodine content of the thyroid gland. In view of the further fact that 50 per cent of the thyroid I_2 may be in the form of diiodotyrosine (Harington and Randall, 1929), the possibility existed that the injected tyrosine was being taken out of the circulation by the thyroid and there stored as diiodotyrosine. Accordingly, we repeated our experiments on four thyroidectomized animals.

The results are summarized in table 3. As in the normal animals, only after 5 grams of tyrosine were injected did phenols appear in the blood, and then only immediately after the injection, at which time between 96 and 98 per cent had apparently disappeared. On taking the next sample, one and two hours later, respectively, in the two experiments, there was no tyrosine to be found in the blood. The rise in blood NH_2 nitrogen was negligible, and in two experiments where this was determined, there was no appreciable increase in urea nitrogen. The blood findings were thus very similar to those observed in the case of the normal animals, and there was no evidence to indicate that any greater amount of tyrosine remained in the blood when the thyroid was absent. It may be inferred that the thyroid is not primarily concerned with the rapid disappearance of blood tyrosine, though the probability that the thyroid may take up this amino acid is not of course ruled out.

In the urine, total N tended to decrease in these experiments, while as a rule NH_2 nitrogen increased slightly. Phenols, except in experiment 2, when 1 gram of tyrosine was administered, increased, but in no case to a considerable extent.

CONCLUSIONS

1. When as much as 5 grams of tyrosine are injected intravenously into dogs, there is practically complete disappearance of the amino acid from the blood within 5 minutes after the injection.

2. The lack of appreciable increase in urea formation indicates that this disappearance is not associated with deaminization of the tyrosine.

3. Not more than 5 per cent of the injected tyrosine appeared in the urine as such or as derived phenols.

4. The evidence indicates that no appreciable percentage of the disappeared blood tyrosine was taken up by the thyroid gland.

BIBLIOGRAPHY

FOLIN, O. 1922. Journ. Biol. Chem., li, 377.
FOLIN, O. AND W. DENIS. 1912. Journ. Biol. Chem., xii, 141.
FOLIN, O. AND H. WU. 1919. Journ. Biol. Chem., xxxviii, 81.
FOLIN, O. AND A. D. MARENZI. 1929. Journ. Biol. Chem., lxiii, 89.
HARINGTON, C. R. AND S. S. RANDALL. 1929. Biochem. Journ., xxiii, 373.
KIECH, V. C. AND J. M. LUCK. 1932. Journ. Biol. Chem., xciv, 433.
LUSK, G. 1912. Journ. Biol. Chem., xiii, 155.
MARINE, D. AND J. M. ROGOFF. 1917. Journ. Pharm., ix, 1.
RAPPORT, D. AND H. H. BEARD. 1927. Journ. Biol. Chem., lxiii, 299.
SHAMBAUGH, N. F., H. B. LEWIS AND D. TOURTELLOTTE. 1931. Journ. Biol. Chem., xcii, 499.
VAN SLYKE, D. D. AND G. M. MEYER. 1913. Journ. Biol. Chem., xvi, 199, 213.

THE RESPIRATORY QUOTIENT OF EXERCISE IN PANCREATIC DIABETES

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In an unpublished experiment performed in 1927, Rapport and Ralli found that in mild exercise and the recovery therefrom, the respiratory quotient of a depancreatized dog remained at the same level (0.71) as it was when the dog was resting. This would indicate that the exercising diabetic animal had no greater tendency to oxidize carbohydrate than the resting one, and that in both cases the source of energy was fat. Grafe and Salomon (1922) and Richardson and Levine (1925) have observed a failure of the R. Q. to rise in the incomplete diabetes of human beings; and in the experiments of Hetzel and Long, also on human diabetics (1926), a rise from the resting level of 0.76 to only 0.80 during exercise was observed. These results would appear to be consistent with the above mentioned argument. Similar results were obtained by Rapport and Ralli (1928) in the phlorhizinized dog.

However, Chaikoff and Macleod (1929) obtained a rise of R. Q. in exercising depancreatized dogs. The exercise was produced by causing the dogs to shiver as a result of exposure to a temperature of 10° to 13°C. in a respiration chamber. The resting R. Qs. were 0.68 to 0.70, while during the first half hour of shivering the quotients varied between 0.66 and 0.85. Later periods of shivering for the most part showed no rise in the R. Q. It is important to note that recovery periods were not obtained, since the respiration chamber was maintained at low temperature throughout the experiment. It was assumed, on the basis of the blood CO_2 content of one animal before and after a comparable shivering period, that the CO_2 content of the dogs whose R. Q. was determined was unchanged, and that hence the rise in R. Q. truly represented an increase in the combustion of carbohydrate. Rapport (1930) pointed out that the validity of this control was questionable.

The experiments here reported were performed with a view to obtain the total gaseous exchange of recovery periods as well as of the exercise periods in a depancreatized dog.

EXPERIMENTAL METHODS. The dog used weighed about 9 kilos and had been depancreatized about 1 month before the experiments were begun.

She was kept during this period on a maintenance diet of beef heart, cracker meal, and raw pig's pancreas, enough insulin being administered to keep the urine almost sugar free. Food and insulin were discontinued at least 48 hours before the experiment. No experiment was performed more than 72 hours after such discontinuance (except in one instance, when 96 hours elapsed) in order to minimize the possibility of the animal failing to recover. On the experimental day the animal was placed in the chamber of a closed respiration system of the Benedict-Homans type, and the resting metabolism determined. Then the dog, who had been previously trained to this,

TABLE 1

Respiratory quotients before, during, and after exercise

EXPERIMENT	DATE	HOURS AFTER INSULIN	WEIGHT	AVERAGE R.Q., PRE-EXERCISE	R.Q. EXERCISE	R.Q., POST EXERCISE ½ HR. PERIODS EXCEPT WHERE NOTED										R.Q., EXERCISE AND RECOVERY
						½	1	1½	2	2½	3	3½	4	4½	5	5½	
	1931		*kilos*														
1	July 9	48	8.1	0.70	0.65	0.69											0.67
2	July 14	48	7.7	0.71	0.75	0.72	0.69										0.72
3	July 15	72	7.5	—	0.70*	0.70		0.73†	0.68								0.70 0.71
4	July 23	48	7.7	0.73	0.80*	0.71	0.68	0.63	0.79†	0.63	0.66						0.70 0.72
5	July 24	72	7.2	0.70	0.76	0.71	0.69	0.69	0.69	0.70	0.70	0.73					0.71
6	Aug. 3	48	7.2	0.71	0.72	0.71	0.67	0.75	0.70	0.70	—	0.73	0.72	0.72	0.69		0.72
7	Aug. 4	72	7.1	0.68	0.74 0.70	0.69	0.73	0.73	0.69	0.71	—	0.72	0.71	0.70	0.71	0.69	0.71
8	Aug. 5	96	6.8	0.69	0.67	0.75	0.72	0.63	0.73	0.67	0.70	0.67	0.72				0.70
9	Aug. 11	60	6.8	0.72	0.77	0.71	0.68	0.71	0.69	0.77	0.71						0.72
10	Aug. 12	84	6.7	0.72	0.74	0.73	0.73	0.77	0.70	—	0.72						0.73
Average				0.71	0.73												0.71

Dog 21. Depancreatized June 11, 1931. Weight 9.1 kilos. Hysterectomy performed and 7 fetuses removed at time of pancreatectomy.

* First exercise period.

† Second exercise period.

was caused to run on a horizontal treadmill which had been introduced into the chamber. The exercise consisted of 14 minutes' running at about 1.6 miles per hour. The animal was trained to lie down immediately after stopping the treadmill, and the gaseous exchange of the period ending 30 minutes after the beginning of exercise obtained (thus allowing time for stabilization of conditions within the apparatus). Subsequent half-hour recovery periods with the dog at rest were then determined. In two of the earlier experiments the animal was again exercised after a short recovery period, but this was discontinued when it appeared, as will be shown later, that the recovery in the depancreatized dog is more prolonged, or at least more irregular, than in the normal animal.

Blood sugar and CO_2 content, and urinary sugar and nitrogen were determined in the majority of cases before the experiment and immediately after removing the animal from the apparatus. The accuracy of the apparatus was tested by five alcohol checks performed during the course of the experiments. The respiratory quotients obtained were as follows: 0.666, 0.661, 0.658, 0.665, and 0.661, averaging 0.662, the maximum error being 1.4 per cent, or less than 0.01 in the R. Q.

EXPERIMENTAL RESULTS. Upon consulting table 1 it will be seen that the average resting respiratory quotient of the dog was 0.71. In six of the twelve experiments, there was during the exercise period an appreciable rise in the R. Q., which in one case went to 0.80. In the earlier experiments a prolonged recovery period was not followed, as from the previous experience in this laboratory with exercise of such character in normal dogs, it was anticipated that recovery would be complete within an hour after the exercise. As stated before, in two experiments second exercise periods were run after a short recovery on this assumption (expts. 3 and 4). In these early experiments the rise during the exercise period, when it occurred, was compensated by a fall during the recovery periods, so that the total R. Q. of the exercise and recovery was essentially the same as in the resting periods before exercise, as will be seen in table 1, experiments 1, 2, 3, and 4.

In view of the fact, however, that in each of these experiments the R. Q. obtained in the final recovery periods was still lower than in the pre-exercise periods, it was deemed advisable to continue the observation of recovery for a longer period. In the later experiments, therefore, this was done for a duration of 3 to $5\frac{1}{2}$ hours after the cessation of exercise. It will be observed in table 1 that in experiment 6 no appreciable deviation of the R. Q. occurred at any time during the experiment. In another instance (expt. 8) there was actually a fall in the quotient during the exercise period, followed by a rise in the first "recovery" period, the average of the two being practically that of the pre-exercise resting periods. In other cases a slight rise of R. Q. in the exercise period was observed; and in still others a greater one—as much as 0.07. During subsequent recovery periods a certain irregularity is to be observed in many experiments. In some cases the fluctuation in adjacent periods is considerable. For example, in experiment 6, R. Qs. of 0.67 and 0.75 were obtained in the periods 1 and $1\frac{1}{2}$ hours after exercise, respectively; and in experiment 9, quotients of 0.69 and 0.77 in the periods 2 and $2\frac{1}{2}$ hours after exercise.

In this type of experiment with the diabetic dog, therefore, it is practically useless to calculate an excess quotient for exercise and recovery, for the end of the recovery is almost impossible to determine with accuracy. One would be driven to use all of the periods obtained after exercise as representing recovery, and as Gemmill (1931) has well pointed out, "the

excess respiratory quotient for long recovery periods is a mathematical abstraction." Nevertheless, an examination of the data will show that over the whole period of exercise and the following periods the average respiratory quotients were almost identical with the pre-exercise quotients. This is true for shorter as well as longer observations of recovery, in all cases compensatory falls in the R. Q. balancing any rise that occurred. The observed fluctuations were as great late in recovery as during and immediately following the exercise itself. The conclusion seems inescapable that they were due, not to oxidation of carbohydrate, but to

TABLE 2

Chemical findings

EXPERIMENT NUMBER	DATE	PRE-EXPERIMENTAL PERIOD					EXPERIMENTAL PERIOD				
		Hourly N of urine	Hourly sugar in urine	D/N	Blood sugar	Blood CO_2	Hourly N of urine	Hourly sugar in urine	D/N	Blood sugar	Blood CO_2
		mgm.	*mgm.*		*mgm. per cent*	*vol. per cent*	*mgm.*	*mgm.*		*mgm. per cent*	*vol. per cent*
1	7/ 9/31	—	—	—	235	32.6	270	760	2.8	263	27.1
2	7/14/31	212	1780	8.35	405	35.3	262	1660	6.35	322	33.0
3	7/15/31	193	775	4.02	242	25.1	185	658	3.55	327	—
4	7/23/31	574	363	6.28	303	—	227	1141	5.04	329	—
5	7/24/31	172	774	4.5	304	28.7	170	493	2.89	338	26.1
6	8/ 3/31	—	—	—	—	45.2	147	600	4.08	—	—
7	8/ 4/31	114	446	3.90	340	—	192	621	3.23	254	—
8	8/ 5/31	—	—	—	243	—	—	—	2.55	294	—
9	8/11	—	—	—	325	39.2	—	—	5.5	250	38 4
10	8/12	—	—	4.8	239	—	—	—	3.9	224	—

fluctuations in the amount of CO_2 driven off from the body as a result of fixed acid accumulation.

In table 2 will be seen a summary of the chemical findings. Though always high, the blood sugar shows considerable fluctuation between the beginning and the end of the experiments. The D/N also fluctuated considerably, as would be expected in individual observations over short periods. Where observed, there was no noteworthy difference between the blood CO_2 content before exercise and at an interval of from 3 to 5 hours after exercise. As the respiratory quotients before and after exercise were, on the average, the same, this was to be expected.

The findings as a whole are entirely consistent with the belief that the completely diabetic animal does not utilize carbohydrate in exercise.

We wish to express our indebtedness to Dr. David Rapport, at whose suggestion and under whose kind direction this work was conducted.

SUMMARY

A depancreatized dog was exercised on a treadmill in a respiration apparatus, food and insulin having been withheld at least 48 hours before individual experiments. While in some cases there was an appreciable rise in the respiratory quotient during exercise, this, when it occurred, was always compensated for by a fall in the subsequent recovery periods, so that the average exercise and recovery quotient was the same as the pre-exercise quotient (about 0.71).

It is concluded that the depancreatized dog does not oxidize carbohydrate in muscular exercise.

BIBLIOGRAPHY

CHAIKOFF, I. L. AND J. J. R. MACLEOD. 1929. Quart. Journ. Exper. Physiol., xix, 291.

GEMMILL, C. L. 1931. This Journal, xcviii, 135.

GRAFE, E. AND H. SALOMON. 1922. Deutsch. Arch. f. Klin. Med., cxxxix, 369.

HETZEL, K. S. AND C. N. H. LONG. 1926. Proc. Roy. Soc. (B), xcix, 279.

RAPPORT, D. 1930. Physiol. Rev., x, 349.

RAPPORT, D. AND E. P. RALLI. 1928. This Journal, lxxxv, 21.

RICHARDSON, H. B. AND S. Z. LEVINE. 1925. Journ. Biol. Chem., lxvi, 161.

Addendum. Since this paper was written, an article has appeared by Chambers, Kennard, Pollack, and Dann (Journ. Biol. Chem., 1932, xcvii, 525), reporting experiments in which depancreatized dogs were made to exercise either by epinephrine injections, or by electrical stimulation under amytal anesthesia. The authors come to substantially the same conclusion as the one made in this paper. For details, the reader is referred to the original article.

POTENTIALS PRODUCED IN THE SPINAL CORD BY STIMULATION OF DORSAL ROOTS

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Electrophysiological methods have had extensive use in studies of spinal cord activity in connection with the afferent and efferent neurones, but very little use has been made of them in the investigation of what goes on in the cord itself. An experimental survey of the situation at once rendered it apparent not only that large potentials are generated in the cord, but that they manifest a wealth of detail of such a nature as to give great promise of shedding new light on the problems of reflex physiology. The results of this survey, as far as it has been completed, are therefore presented in this paper.

METHOD. All the experiments were performed on cats. The lower part of the spinal cord was exposed under ether anesthesia. After the dura was opened, one of the post-thoracic dorsal roots—usually the seventh lumbar, but sometimes one of the roots adjacent to it—was severed at its exit from the spinal canal and laid on a pair of electrodes for stimulation, the cathode being next to the cord. The stimulation was effected by break shocks from a Porter coil with the core in place. Leads were made from the dorsum of the cord by means of camel's hair brushes moistened with Ringer's solution and transfixed with chloride-coated silver wires. They were 2 to 3 cm. apart in the direction of the axis of the cord, and connected to a cathode ray oscillograph in such a way that a deflection upward in the records means that the posterior lead (nearer to the root) was negative to the anterior. The experiments were performed either under various depths of ether anesthesia or without anesthesia following decerebration and transection of the cord in the lower thoracic region.

THE SPIKE POTENTIAL. When a dorsal root is stimulated the first event to be recorded from the cord is a spike which always appears in triphasic form (fig. 1), as would be expected if conduction is taking place in a thread of active tissue surrounded by a mass of inactive tissue (Craib; Bishop and Gilson). Measured between the two minima the spike has a duration of 0.5σ, which, allowing for the distortion introduced by the triphasicity, is obviously the duration of the axon action potential of a mammalian A

fiber at body temperature (Gasser, 1928). The form of the recorded response varies with the distance of the lead from the point of entrance of the root. When the lead is near the root the first phase is small and the third large, but when the lead is moved up the cord away from the root, the first phase increases and the third phase decreases. The explanation of this change is simple. The artifacts (first and third phases) are due to potentials developing elsewhere than at the active lead. When the lead is near the root, the impulse comes under the first or active lead so promptly that the pre-spike artifact is small; but the spike is followed by a large diphasic artifact produced by activity higher up in the cord. When the lead is far from the root, the conduction time between the root and the lead is longer and the pre-spike artifact persists throughout this period. On the other hand the diphasic artifact becomes smaller because, as will be elucidated in the following paragraph, the height of the spike itself decreases as the lead is moved away from the root, and there is therefore less potential produced in the cord anterior to the lead to be recorded as an artifact.

Fig. 1. Characteristic triphasic record obtained from the spinal cord, following stimulation of the 7th lumbar dorsal root. Threshold stimulation was used and the lead was 8 mm. above the midpoint of the entrance zone of the root. The spike therefore approaches axonal dimensions. ↓ marks stimulating shock. Time in sigmas.

The spike is maximal when the lead is placed opposite the point of entrance of the root. Above the point of entrance the magnitude of the spike falls away along a curve which is illustrated in figure 2. In the experiment cited the stimulus was strong enough to excite all the α and most of the β fibers. Theoretically the form of the curve should vary with the number of fibers active; but in the absence of any systematic investigation of this point, it can only be stated that even when α fibers only are functioning, the spike height falls to a small fraction of its initial value between 3 and 4 cm. above the root. The decrease in area is much less than the spike heights, taken alone, would indicate, as the spike undergoes an increase in duration of the type which in peripheral nerve is interpreted as temporal dispersion. Below the root, the magnitude of the spike falls off rapidly, and it is difficult to follow the wave farther than 6 to 7 mm.

The rate of conduction of the process producing the spike was measured by determining the intervals elapsing between the time of appearance of the spike at the root-level and at various distances higher up in the cord. Conduction-time curves were mapped out in this manner in six cats, and they were in all cases identical in form. The impulse starts up the cord at about 80 meters per second but after traveling 3 to 4 cm. it slows up,

and conduction is continued, as far as it has been followed, at 30 meters per second (fig. 2). These rates were determined in fresh preparations in which the circulation through the cord was active. As the laboratory temperatures were between 30 and 33°C., the cord temperatures were probably not greatly subnormal, and the velocities indicated are therefore not much below the real value. The dorsal root fibers were of course at subnormal temperatures, but the conduction time in the roots does not enter into the determinations.

Discussion. For the sake of simplicity the more sensitive A fibers only are considered. When a dorsal root fiber enters the cord it divides into an ascending and a descending branch. The ascending branch, after giving off collaterals to the grey matter of the same and adjacent segments, is continued upward as an unbranched fiber in the dorsal column. All the

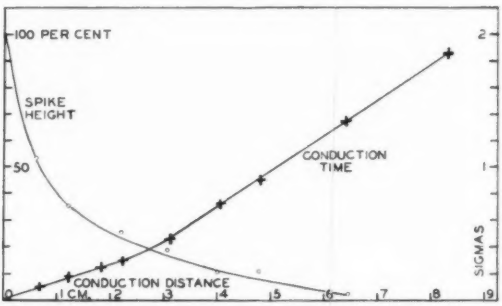

Fig. 2. Conduction in the intramedullary portion of the 7th lumbar root. Distance measured from the point of entrance of the root. Spike height referred to the size obtained at zero conduction. (5/7/32.)

properties of the spike potential can be accounted for on the assumption that what is being recorded is the potential produced in the intramedullary continuation of the dorsal root fibers. The duration of the spike and the initial velocity of the impulse are quite in accord with the values known for the same properties in the extramedullary portions of the axons, and the changes in the magnitude and velocity of the spike can readily be brought into connection with the histology of the cord.

The size of the spike depends upon the amount of active tissue; that is, not only on the fibers constituting the long pathways but also on their collaterals. It is not surprising, therefore, that the spike should be large at first and become progressively smaller as the impulse is continued up the cord, for it is a well-known fact that the number of collaterals is greatest in the segment belonging to the root and becomes progressively less in the adjacent anterior segments. Through the ending of the fibers and col-

laterals in succeeding segments the amount of potential produced is reduced, but the slowing of the velocity of conduction must have another basis. The slow rate of 30 meters per second at which impulses are carried toward the nucleus in the medulla oblongata suggests that the fibers involved are considerably smaller than those which carry the impulses in the lumbar enlargement. Evidence that this is the case is found in a statement by Cajal (1909, I, 302) to the effect that the fibers in the column of Goll have a diameter of 2 to 3μ.

Records of the impulse as it passes through the region of numerous collaterals show a spike of the same duration as in peripheral nerves. This fact is not incompatible, however, with the assumption of occupation of the collaterals by the nerve impulse made in the preceding paragraph. The collaterals can not have a length of more than 2 to 3 mm., and a simple

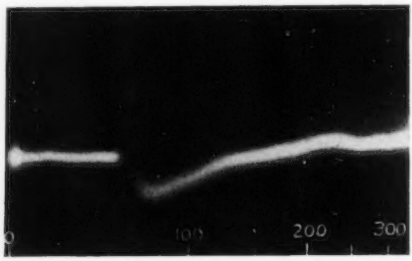

Fig. 3. Response of the spinal cord to stimulation of a portion of the 7th lumbar root. The spike does not show; the upward deflection is the negative wave and the downward one the positive. Time in sigmas.

calculation shows that conduction through them at 80 m.p.s., or even at a much slower velocity, does not increase the duration of the spike at any point sufficiently to bring it out of the range which holds for unbranched nerve fibers.

THE INTERMEDIARY POTENTIALS. The period following the spike is occupied by long lasting potentials (designated as intermediary potentials). In their simplest form they consist of a period of negative potential followed by a longer period in which the lead proximal to the root is positive (fig. 3). In more complicated forms the negative potential appears with two crests, but these forms have not yet been analyzed.

The negative potential is first observable following the diphasic artifact. In the transition from the trough of the artifact to the crest of the negative potential there is often a point of inflection in the curve which indicates that two events are being dealt with—the subsidence of the artifact and overlapping this the development of the negative potential. As will be seen

later, it is possible experimentally to suppress the latter while leaving the former intact. Since the time at which the negative potential commences can not be ascertained, the position of the crest of the potential is measured with respect to the start of the spike. The interval found has a mean value of 2.2σ and there is little variation from experiment to experiment (table 1).

Compared with the height of the spike which precedes it, the size of the negative intermediary potential is by no means small: 40 per cent of the spike height is the smallest value recorded in table 1, and on occasion the negative potential may be higher than the spike itself. In the measure-

TABLE 1

CAT NUMBER	NEGATIVE WAVE			POSITIVE WAVE		INTERVALS* AT WHICH NEGATIVE WAVE OBSERVED TO BE SUBNORMAL	TIME OF APPEARANCE OF POTENTIALS ASSOCIATED WITH REFLEXES
	Time to maximum	Duration	Height (per cent of preceding spike)	Time to maximum	Duration		
	σ	σ		σ	σ	σ	σ
1	1.9	7	40				
2	2.2	8	70	18			
3	2.0	12	40	18			
4	2.0	7.5					
5	2.5	10.7	62				
6	2.4	10	80				
7	2.1	9	56	20–26	>46	36†	2.2
8	2.2	17.6					
9	2.1	11.5		23.5	70	72†	
10	2.2	10.2					3.2
11	2.0	7.5			80		2.3
12				24.5	120	50†	
13	2.6	16	125	25	216	46†	
14	1.8	6.7		15.5	80	63	3.2
15	1.9	10.9	41	16	73		
16	2.2	8.5	82	20	127		3.6

* The intervals do not mark the end of the subnormality.

† Indicates the subnormality to be considerable.

ment of the potentials an arbitrary system is followed; the spike height is measured from the vertical projection of the crest upon the line connecting the troughs of the two artifacts between which the spike is recorded, and the height of the negative potential is measured from the base line established in the record before the stimulating shock is applied to the root. The absolute value of the negative potential as recorded at the surface of the cord varies with the distance of the lead from the root and with the condition of the cord. Potentials as large as 0.5 mv., or more, are frequently encountered.

The duration of the negative wave is measured as the interval elapsing

between the start of the spike and the time indicated in the records by the point at which an extension of the base line cuts the curve. By this method values are found ranging from 7σ to 17.6σ, with the mean at 10.2σ. Strictly speaking these determinations only delimit the time during which the sum of the potentials acting on the lead nearest to the root is such as to keep it negative to the more distant lead. At the point measured the sign changes, and the near lead becomes positive. Now, whatever interpretation may be put on the positive wave, the presumption is that the processes producing it do not start at the end of the negative wave but at some time earlier; and from this it follows that the activity producing the negative wave—which is the subject of our interest—must be in existence longer than the wave itself. In two experiments, both on anesthetized animals,

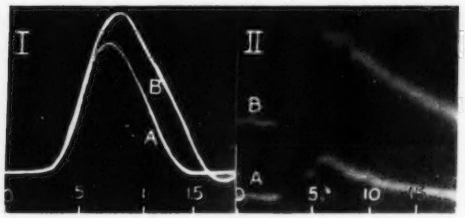

Fig. 4

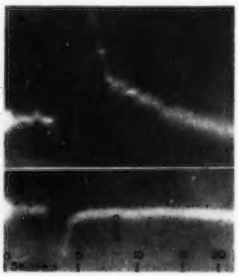

Fig. 5

Fig. 4. Comparison of the response in the sciatic nerve with that in the spinal cord when a dorsal root is stimulated. I. Sciatic nerve, two records retouched and printed superimposed. A, α wave; B, α and a considerable fraction of β. II. Cord. A and B correspond to the same letters in I.

Fig. 5. Effect of asphyxia on the response of the cord of a decerebrate cat to stimulation of a dorsal root. A. Normal response. B. A few minutes later, after the respiration had been stopped by strong ether vapor. The spike was still intact but only the ending of the diphasic artifact is visible in the record.

the positive swing was absent and the negative potential slowly decremented to zero with the end indeterminate.

Relation to strength of stimulation. The negative wave appears in threshold responses of the cord and can be seen when a very small fraction of the α fibers in the root is stimulated. The fiber content of the root which is active during a given cord response can be determined by an alternate lead from the sciatic nerve. As the number of fibers stimulated increases the cord wave grows progressively. Figure 4 shows the increase in the cord response obtained in one experiment when the shock applied to the root was increased from a strength which stimulated the α fibers to one which stimulated a good fraction but by no means all of the β fibers as well.

The increase in this case is fairly symmetrical. More frequently, however, the early part of the wave increases more than the later part, as in figure 9.

Negative intermediary potential and asphyxia. One of the most characteristic features of the wave is its absolute dependence upon a continuously available supply of oxygen. Cessation of the circulation for a time of the order of one minute wipes out the wave completely, and it is greatly reduced when the circulation is impaired. Experimental demonstration of the fact can be made by stopping the artificial ventilation of a spinal animal or, as was the case in the instance illustrated in figure 5, by narcotizing the respiratory center. Asphyxia of the negative wave is so differential with respect to the spike that the latter may retain its full size at a time at which the former has completely disappeared. Prolonged asphyxia later reduces the spike also.

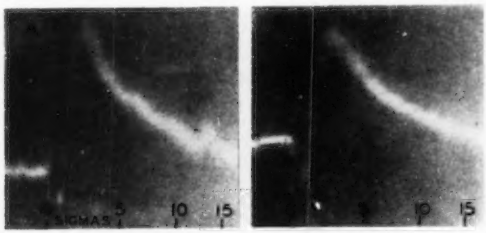

Fig. 6. Effect of narcosis on the response of the cord of a spinal cat to stimulation of a dorsal root. A, without narcosis; B, in very deep narcosis.

Negative intermediary potential and narcosis. The wave is present when the animals are so deeply anesthetized that the strongest shocks fail to elicit a reflex. No relation has been seen between its duration and the depth of narcosis, and unless accompanied by asphyxia narcosis seems to have very little effect on its size. In one experiment a spinal cat was subjected to artificial ventilation through an ether bottle so that oxygenation of the blood could take place after the respiratory center was paralyzed. The response of the cord to stimulation of a dorsal root was compared with the response previously obtained in the same preparation, unanesthetized. As shown in figure 6, a depth of narcosis greater than any that can be reached in a spontaneously breathing animal resulted in but a small decrease in the wave. The decrease may very well be attributed to the state of the circulation as, soon after the second record was taken, the circulation became very weak and the cord response decreased rapidly.

Effect of temperature on the negative intermediary potential. Observations were made of the potential after the cord had been cooled by pouring cold Ringer's solution over its surface. The duration of the potential was thereby greatly prolonged.

Response of the cord to two stimuli applied to the same root. In a series of experiments two break shocks were applied to a dorsal root in rapid succession. The second shock was usually strong enough to stimulate most of the *A* fibers, and the first shock was somewhat stronger. In the instances in which the animals were spinal and unanesthetized it was convenient to keep both the shocks below the threshold of the flexion reflex. When the two shocks were close enough together the second intramedullary spike disappeared; on reappearance, as the separation of the shocks was progressively increased, it was first subnormal in size, then it returned to normal over a curve very similar to the one known for peripheral nerve. In fact the refractory period was undoubtedly determined by the root, as the latter was at a temperature below that of the cord. The cord curve could be considered as the root curve modified by conduction between the stimulus and the lead and by the effect of the increased temperature of the intramedullary portion of the fibers. In a typical experiment the earliest second spike appeared at an interval of 1.3σ after the first one; and when the interval was 2.8σ the spike had its normal size.

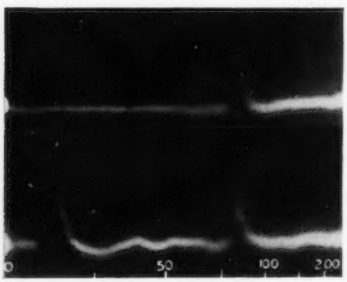

Fig. 7. Two responses of the cord at an interval of 72σ. The upper record shows the response to the testing shock when acting alone. Cat under light ether anesthesia. The small waves in the lower record are fortuitous. Time in sigmas. (5/18/32.)

The earliest recorded spikes, which in the tracings are just after the crest of the negative intermediary wave of the first response, always seem to be succeeded by some negativity of their own. Consequently, even though it be hardly more than a trace in the case of the earliest reponses, there is a resultant increment in the total area of the negativity. For the second negative intermediary wave to reach its full magnitude a much greater shock separation is required than is necessary for the spike. The relation which seems to be necessary for the attainment of normal size is that the wave be set up after the first positive wave has subsided. The curve of recovery has not yet been worked out, but in table 1 intervals are noted at which the wave is still subnormal. Figure 7 illustrates the amount of subnormality in one cord at 72σ.

In two experiments the second negative wave reached full height when it was set up during the first one and in both experiments no positive wave was evident. The necessary conditions for this type of response are unknown, however; so it has been impossible to investigate further this interesting variation. The two experiments were performed under anes-

thesia, but the other type of recovery is the rule in anesthetized as well as in unanesthetized animals.

Response of the cord to two stimuli applied to different roots. These experiments differ from the preceding in that there is no refractoriness as far as the spike is concerned, and the combination can be studied at all intervals. They also have another important significance. The stimuli were applied either to two separate strands of the 7th lumbar, or one to the 7th lumbar and the other to the 1st sacral. If the activity remain throughout in different pathways, simultaneous stimulation of the two roots should result in a simple addition of the two component responses as they exist separately, and previous stimulation of one root should have no effect on the activity induced by stimulation of the other; if, on the other hand, the

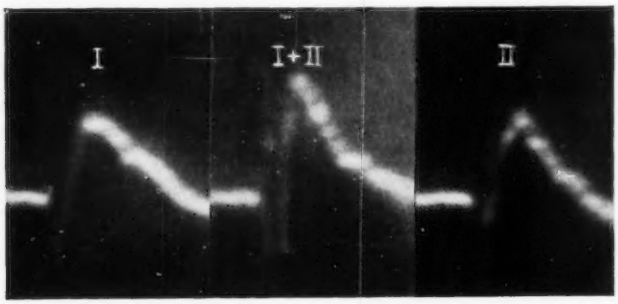

Fig. 8. Effect of simultaneous stimulation of two strands of the 7th lumbar root. Lead 3 mm. above the anterior strand. Cat spinal and decerebrate. Shocks submaximal. I, stimulation of one strand; II, stimulation of the other; I and II, stimulation of both together. (5/27/32.)

pathways converge, the foregoing expectations will not be fulfilled. A decision between the two possibilities can therefore be obtained experimentally.

Three experiments were performed with an identical outcome. A part of one of them is reproduced in figure 8. The 7th lumbar root was divided into two parts. Stimulation of one part with a submaximal shock gave record I; stimulation of the other, record II. When the two were stimulated simultaneously the spikes added; but as can be seen in the middle curve of the figure the area of the negative wave was much less than the sum of the separate responses. When the two divisions were stimulated in succession, the effect of the first stimulus was to decrease the negative wave of the second response in a manner quite like that just described for two stimuli applied to a single root. The situation was no different when the stimuli were applied to adjacent roots, as L7 and S1; but two experi-

ments, in which paired roots were stimulated, resulted in simple addition if the shocks were simultaneous, and brought out no evidence of antagonism if the shocks were separated. Under conditions in which homolateral roots would have exerted mutual effects on each other, such effects were absent for opposite roots. It does not follow from this that a crossed influence can not be evoked, but rather that such an influence if it occurs needs other experimental conditions, presumably on account of the more complicated neurone chain involved.

The conclusion to be drawn from the foregoing experiments is that the spike belongs to the axon of the first neurone, but that the negative potential is generated at some point farther down stream, for example, in the terminations of the first neurone, in the internuncial neurones, or in the motor cells. The last mentioned are the most accessible to experimentation.

Activity of the motor cells as led from the dorsum of the cord. With the initiation of reflexes the motor cells must become active, therefore one mode of approach is to watch for the change in the electrical pattern which takes place simultaneously with the development of the flexion reflex. In deep narcosis the negative wave maintains a perfectly smooth contour at all strengths of stimulation; but in light narcosis and in the unanesthetized spinal animal the contour is smooth only when induced by weak shocks; as soon as the reflex appears, a wavy indentation occurs in the outline just after its maximum, as indicated by the arrow in figure 9. The time of beginning of the indentation after the start of the spike has been measured in a number of experiments and the findings included in table 1. The times are such as to identify them with the reduced reflex time of Jolly, Forbes and Gregg, and Eccles and Sherrington (1931a); only they are slightly more reduced as intramedullary conduction is not included. The indentation gives the impression that it is made up of a number of short waves out of phase, possibly of spikes. In experiments on the spinal animal stronger shocks bring in a large number of spike-like potentials and the picture becomes so complicated, particularly if an after-discharge is set up, that strong shocks have been avoided.

The foregoing experiments show an association of spike-like potentials with reflex activity. In this activity the motor cells must be active, but it is possible that the potentials may be derived from the motor roots rather than from the cells themselves. No new prolonged potentials were observed, but on the possibility that such a potential might be obscured on the background already present, the cells were stimulated antidromically. This is a method devised by Denny-Brown, who argued—we believe correctly—that conduction from the nerve fiber should go backward over the nerve cell. In the face of irritability of the cells proven by active reflexes, antidromic stimulation brought about but one result, a simple triphasic

spike (fig. 10). If the negative potential set up by impulses in the normal direction is in the motor cells, it should be led equally effectively from the back of the cord when set up antidromically. The inference therefore is that the motor cells are not the source of the potential. It is possible that these cells can not be excited from the roots, and that all the potential recorded was from the roots rather than the cells; but the duration of the raised neurone threshold observed by Denny-Brown and by Eccles after "backfiring" on the motor cells indicates almost surely that such is not the case.

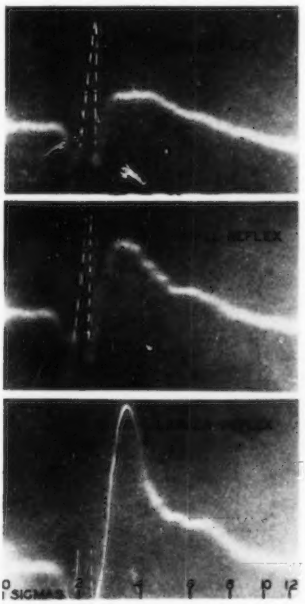

Fig. 9

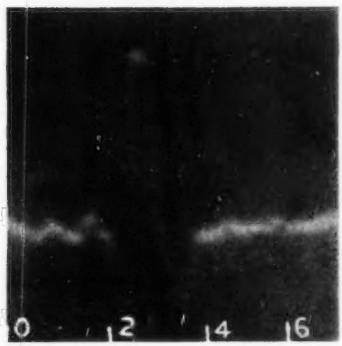

Fig. 10

Fig. 9. Potentials appearing simultaneously with the flexion reflex discharge.

Fig. 10. Record obtained from the spinal cord when the sciatic nerve was stimulated after all the dorsal roots had been cut. Time in sigmas.

Two further observations indicate that the negative potential is generated elsewhere than in the motor cells. First, as already noted, the potential persists in depths of narcosis in which reflex excitation of the cells is impossible. Second, no interference takes place between an antidromic spike and the negative potential when these are set up in juxtaposition, as would be expected if the two events occurred in the same structure and the refractory state could be produced by stimuli from either direction. The

potential changes occurring in the motor elements are merely written on the negative potential as a base line. In the performance of the latter experiment the motor roots were stimluated by single maximal shocks applied to the sciatic nerve after the sensory roots had been cut intradurally, and the antidromic impulses were opposed by impulses set up in the 7th dorsal root isolated from the nerve.

THE POSITIVE POTENTIAL. The variations in the recorded dimensions of this wave are given in table 1. On the average it reaches its maximum in 20σ and becomes indefinite after 80 to 100σ, sometimes longer. From a technical standpoint the principal interest in the wave is why it is positive. Although the records were made with a condenser-coupled amplifier, the positivity can not be due to amplifier distortion. The duration of the wave is short compared with the curve of discharge of the amplifier condensers and, since the area of the wave is much larger than that of the preceding negative wave, the wave itself can not be ascribed to filling of the condensers during the period of negativity. The explanation must be sought in the cord itself.

Positivity may mean that the tissue under the active electrode has become more polarized as heart muscle is supposed to be in the usual interpretation of the Gaskell phenomenon, or that the other electrode has become more negative. In the cord we are dealing with a negative wave followed by a positive one. This combination, which is frequently encountered in electrophysiology, is most often due to a propagated disturbance, the center of negativity being first nearer to one electrode and then nearer to the other. The cord intermediary potentials will first be examined to see if they fit into this category.

A reference back to figure 2 brings out the fact that the time in which the activity is spreading through the cord is very short in comparison with the duration of the potentials which are set up. In other words, the period of invasion is short in comparison with the period of occupation, which means that the change in the sign of the potential can not be due to conduction in the direction of the long tracts of the cord. To demonstrate that the positivity is due to negativity at the lead farther from the root, it would be necessary to show a more rapid subsidence of activity under the proximal lead than under the more distant one. Experimentation shows that this does not occur: if the cord be examined with three electrodes placed at distances repectively of 1, 3 and 5 cm. above the root, the potentials led from the first pair have the same time-relations as those from the second pair; the only difference is that the latter are smaller (compare fig. 2). Therefore a comparison is made at all times between a potential at the near electrode and a smaller one of the the same sign at the far electrode, from which it follows that the sign of the potential under the active electrode must be due to the nature of the process under it. Even so it

does not follow that any structure has become more positive. If the source of potential were elongated in form, oriented in the dorso-ventral line and active (negative) at the ventral end, the effect on the electrodes above it would be to make the nearer one positive (fig. 11). On this basis the difference between the positive and negative potentials would be in the orientation of the structures producing them. It might be argued that the change in sign from negative to positive might be due to conduction of the impulse along such a structure, but the duration of the negative wave in comparison with the reflex time is very much against such an hypothesis.

Discussion. The most interesting feature of the cord electrogram is the prolonged potentials which, provided there is sufficient depth of narcosis, present a perfectly smooth contour. Their duration and freedom from oscillations place them in the group of potentials which have from time to time been described as occurring in the central nervous system. Potentials of a duration longer than the spikes of peripheral nerve impulses have been recorded in recent years from the cerebral cortex by Práwdicz-Neminsky, Berger (who reviews the older literature), Bartley and Newman, and Fischer. As recently described by Bishop and Bartley, the waves in the rabbit have a duration of 30 to 100σ and are free from oscillations. Waves similar in type have been derived from the optic lobes of the gold fish by Adrian and Buytendijk. They have the rhythm of the respiration and durations up to $\frac{1}{4}$ second. All the authors are in agreement in holding that the waves in question are long potential changes rather than a summation of shorter ones.

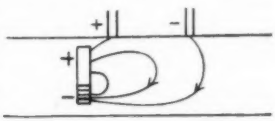

Fig. 11

The similarity of the cord potentials to the after-potential of nerve demands a comparison of the qualities of these two phenomena. Such a comparison is only possible, however, if the properties of the after-potential in frog nerve are taken as characteristic of after-potentials in general, as little is known about the after-potential in mammalian nerve except that it exists in excised nerve (Davis, Pascual and Rice) and is probably negligible in size or absent in the nerves as they exist in the body.

In the case of both the nerve after-potential and the negative cord potential, the spike is followed by a long low potential which rises to a maximum and then declines. Both are differentially sensitive to asphyxia; and, like the cord potential, the full area of the after-potential can not be reproduced unless the two responses are separated by a considerable period (Zotterman). The similarity between the two is so great that the after-potential-like nature of the cord potential can not be denied, but when their properties are considered quantitatively, the resemblance is much less close than the qualitative description implies. The cord potentials are many times larger than the after-potential and are much more differentially

sensitive to asphyxia. In addition, another fact speaks strongly against the interpretation of the cord potentials as having the nature of after-potentials. If the cord potentials were after-potentials occurring in the first neurone, then when two root branches are stimulated simultaneously, the result obtained should be simply the sum of the two separate components, whereas, as has been demonstrated, the result is much less than the sum. Clearly, if we are dealing with an after-potential it can not be in the first neurone; and if we assume that it is in the second neurone, it is necessary to make some secondary assumption to account for the fact that the spike which should initiate the potential is not recorded.

We have presented strong evidence that the prolonged waves in the cord are produced beyond the primary neurone. Motor cell activity alone does not produce them, and unless we make the improbable assumption that, when stimulated in the normal direction, these cells build up a potential before they become active, they can not be involved. Such an assumption would be contrary to what happens in peripheral nerve, as Erlanger and Blair have found that there is no potential sign of subthreshold excitation. The burden, therefore, seems to fall on the internuncial neurones. These consist of three parts: the cell body, the axon, and the *pieds terminaux*. Of these the axon is the least probable source because the nature of axon potentials is known and does not fit. Both Adrian and Buytendijk and Bishop and Bartley have argued for the cells (which include the dendrites), but the argument would hold with equal force for the *pieds terminaux*. Differentiation between the two could theoretically be obtained by a study of dorsal root ganglia which contain ganglion cells but no synapses, for the impulse in all probability is normally conducted to the cells in spite of the lack of necessity for such conduction in the functioning of the neurone.

One of the most interesting problems in cord physiology is the minimum number of neurones involved in a reflex. Histologically the largest dorsal root fibers have been described as making direct endings on the motor cells (e.g., Winkler), but this is a point on which there is not complete agreement among histologists; for instance, Hoff with his new method finds that no endings on motor cells degenerate when the dorsal root is cut. As between a two-neurone arc and a three-neurone arc the electrical picture supports the latter, but the former can not be eliminated as long as assumptions reconcilable with the facts can be made. In a two-neurone arc the only place where the cord negative wave could be produced, as far as the arc itself is concerned (the same fiber might through a collateral excite an internuncial neurone), would be in the terminations of the primary neurone. In this event it would be necessary to postulate some form of mutual antagonism between simultaneously active terminations on the motor cells in order to explain the failure of the two roots to produce an effect

equal to the sum of the effects of each one singly. While such a postulate is not in violation of any facts, it has the weakness of not having any basis in the known properties of nervous tissue.

It is because all the evidence so far available points to a location of the potentials between the first and the last neurone that they have been designated intermediary potentials.

All investigators who have pondered on the possible explanation of why the spinal cord reflexes have the qualities which have been observed, have seen the necessity for some element in the complex having a longer duration than the impulse in peripheral nerve. Sherrington makes use of the expression "central excitatory state" (c.e.s.) to designate the excitation mechanism in the nervous system, and with his colleagues he has performed many ingenious experiments bearing on its nature and location. In the definition of the state, however, its nature and location are left indefinite. Arguments in favor of a chemical interpretation of the state have been adduced by Fulton. Bremer considers summation in the reflex arc as analogous to peripheral summation: a residual excitation corresponding to the local process of Lucas is left by the first impulse and to this the excitation brought by the second impulse is added. Forbes, Davis, and Lambert have suggested that a persistent effect like the "retention of action current" of Levin may possibly be the basis for the summation of successive nerve impulses. Adrian and Bronk make use of a "more lasting" excitatory state in explaining the rhythm of discharge in motor fibers, and Adrian and Buytendijk note the possible significance of the potentials obtained from the optic lobes of the gold fish in connection with the lasting states in the nervous system.

A discussion of the foregoing suggestions is outside the province of this paper. The common point to be drawn from them is the duration of the event postulated. Perhaps the most interesting statement of the view occurs in a paper by Eccles and Sherrington (1931a, p. 527) to the effect that "at some central part of the reflex arc there must be a place where a nerve impulse gives rise to a condition which endures for some time before setting up an impulse in the next part of the central pathway." One of the prolonged potentials we have recorded may very well be the potential-sign of such a condition; in fact, the statement is almost a prediction of the existence of such a potential.

The intermediary potentials can only become useful in spinal cord physiology provided they can be specifically associated with other manifestations of activity; therefore several associations which may possibly turn out to be valid are mentioned.

When two peripheral afferent nerves are stimulated, the tension reflexly produced in a muscle is more than is obtained when one of the nerves is stimulated alone, but much less than the sum of the tensions evoked by

stimulating the nerves separately (Cooper, Denny-Brown and Sherrington). The same is true for the area of the negative intermediary potential.

In the experiments of Eccles and Sherrington (1930), when a flexion reflex was induced by stimulation of two afferent nerves, summation occurred at intervals up to 8 to 15σ in those instances where the optimum was at simultaneity (figs. 1, 7, 8, 12, 16, 20). These intervals are close enough to the durations of the negative potential to suggest a relationship.

If the size of the flexion reflex evoked by the second of two shocks be compared with the isolated reflex similarly produced, it is often found to be smaller for a considerable period. Judged by the action potential in the nerve, it was still subnormal at 50σ in the majority of the experiments of Forbes, Querido, Whitaker and Hurxthal; and repeated stimuli were found by Gerard and Forbes not to yield full sized potentials even when spaced 0.8 second apart. The tension attributable to the second shock was found by Eccles and Sherrington (1931b) to vary according to the conditions of the experiment. In their third type, recovery did not take place until the interval was at least 80σ and sometimes more than 400σ. Their figure 5 shows that the reflex produced by the second shock was minimum at 20σ; and recovery was incomplete at 120σ. The similarity of this curve with the positive intermediary potential deserves consideration. Moreover, inasmuch as Eccles and Sherrington find that periods of unresponsiveness longer than 80σ, and possibly some periods shorter than these, are due to inhibition the problem is put before us of whether the positive potential may not be connected with the process responsible for inhibition.

At the present time at the center of interest in the field of reflex physiology are the two states designated by Sherrington as the c.e.s. and the c.i.s., two concepts about which the experiments of the Oxford School are built. Whatever bearing the intermediary potentials may have on the evolution of these concepts, we feel that speculation on our part in this regard is, in the present state of our knowledge, as apt to bring confusion as clarity, and it is not intended that unwarranted inferences in this respect be drawn from the illustrations cited above. Certainly any notions of identity should be avoided. For instance, weighty evidence has been derived by the Oxford school in favor of the location of c.e.s. in the motoneurone, while to the best of our knowledge the intermediary potentials are produced one neurone farther up stream; therefore, as the matter stands at the moment, the relationship could only be that of the induction coil to the local excitatory process of Lucas.

SUMMARY

The potentials which develop in the spinal cord of the cat after threshold or submaximal stimulation of dorsal root fibers are recorded from electrodes placed on the dorsum of the cord.

The first event is a spike attributable to the intramedullary course of the dorsal root fibers. It has the duration which holds for *A* fibers in peripheral nerve.

When the 7th lumbar root is stimulated the spike is largest at the entrance of the root and falls off rapidly during the first 3 to 4 cm. The initial velocity is 80 m.p.s.; but above the lumbar enlargement the impulse is conducted at 30 m.p.s. Below the root the spike can be traced for 6 to 7 mm.

Leads from the lumbar enlargement show that the spike is immediately followed by two potential waves with a smooth contour: one recording in the negative direction with a crest time of 2.2σ and an apparent duration of 10.2σ (all figures are averages); and another recording in the positive direction with a crest time of 20σ and a duration of 80 to 100σ. These waves are called, respectively, the positive and negative intermediary potentials.

When reflexes develop additional potentials appear which are spike-like in character.

The negative intermediary potential has the following properties: it appears after threshold stimulation and grows as the number of fibers stimulated increases; it is prolonged by cooling, and is very sensitive to asphyxia, but resistant to narcosis.

When a root is stimulated by two shocks, the second intramedullary spike recovers over a curve determined by the root fibers; but the negative intermediary potential is restored much more slowly, not reaching its full magnitude until near the termination of the positive potential of the first response. Except for the refractoriness of the spike the same phenomena can be evoked when the shocks are applied to separate roots.

Simultaneous stimulation of adjacent homolateral roots results in a negative wave much smaller than the sum of the two component waves, indicating that convergence has taken place on the afferent side of the site of production of the wave.

Leads from the dorsum of the cord record only a spike when the anterior horn cells are stimulated antidromically.

The information available indicates that the most probable origin of the intermediary potentials is in the internuncial neurones.

BIBLIOGRAPHY

Adrian and Bronk. 1929. Journ. Physiol., lxvii, 119.
Adrian and Buytendijk. 1931. Journ. Physiol., lxxi, 121.
Bartley and Newman. 1930. Science, lxxi, 587.
Berger. 1929. Arch. f. Psychiat. u. Nervenkrankh, lxxxvii, 527.
1931, Ibid., xciv, 16.
Bishop and Bartley. 1932. Proc. Soc. Exp. Biol. and Med., xxix, 698.
Bishop and Gilson. 1929. This Journal, lxxxix, 135.
Bremer. 1929. Compt. Rend. Soc. Biol., cii, 332.

CAJAL. 1909. Histologie du système nerveux. Paris, i, 302.
COOPER, DENNY-BROWN AND SHERRINGTON. 1927. Proc. Roy. Soc. B, ci, 262.
CRAIB. 1928. Journ. Physiol., lxvi, 49.
DAVIS, PASCUAL AND RICE. 1928. This Journal, lxxxvi, 706.
DENNY-BROWN. 1928. Proc. Roy. Soc. B, ciii, 321.
ECCLES. 1931. Proc. Roy. Soc., B, cvii, 557.
ECCLES AND SHERRINGTON. 1930. Journ. Physiol., lxix, 1.
1931a. Proc. Roy. Soc. B, cvii, 511.
1931b. Proc. Roy. Soc. B, cvii, 535.
ERLANGER AND BLAIR. 1931. This Journal, xcix, 108.
FISCHER. 1932. Pflüger's Arch., ccxxx, 161.
FORBES AND GREGG. 1915. This Journal, xxxvii, 118.
FORBES, QUERIDO, WHITAKER AND HURXTHAL. 1928. This Journal, lxxxv, 432.
FORBES, DAVIS AND LAMBERT. 1930. This Journal, xcv, 142.
FULTON. 1926. Muscular contraction and the reflex control of movement. Baltimore, 349.
GASSER. 1928. This Journal, lxxxiv, 699, fig. 2, I.
GERARD AND FORBES. 1928. This Journal, lxxxvi, 186.
HOFF. 1932. Journ. Physiol., lxxiv, 4P; 1932. Proc. Roy. Soc. B, cxi, 175.
JOLLY. 1911. Quart. Journ. Exper. Physiol., iv, 67.
LEVIN. 1927. Journ. Physiol., lxiii, 113.
PRÁWDICZ-NEMINSKY. 1925. Pflüger's Arch., ccix, 362.
SHERRINGTON. 1929. Proc. Roy. Soc. B, cv, 332.
WINKLER. 1918. L'anatomie du système nerveux. Haarlem, i, 187.
ZOTTERMAN. 1928. Journ. Physiol., lxvi, 181.

STUDIES ON THE INDIRECT MEASUREMENT OF BLOOD PRESSURE

II. A Three-Bag System for Measurement of Blood Pressure in Man

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In the first paper of this series (1932) it was shown that oscillatory methods gave the greatest promise for the exact analysis of blood-pressure levels in man, and that the "formo-ozsillatorisch" criteria of von Recklinghausen appeared to provide the most reliable indices. With ordinary oscillatory methods these criteria could often but not always be demonstrated in records from the human arm, but were much less frequently demonstrable in records from the thigh of a dog; consequently it appeared important to obtain such records with greater certainty and accuracy. Increasing the natural frequency of the recording system by reducing the size of the receiving bag on the arm improves the apparatus, but accuracy cannot be obtained unless the pressure is applied over a relatively large area. Consequently a small rubber bag was enclosed within a larger compressing unit, and the problem became one of adequate insulation of the smaller bag from the pulsations of the larger. Ultimately the insertion of an intermediate insulating air-bag between the other two bags appeared to offer the best solution. It is also important to be able to utilise continuous inflation and deflation and yet prevent any serious distortion of the waves as the result of the leak in the system. This was achieved by connecting the two inner bags to the oscillometer as a closed system compressed by the outer bag, so that the air movements in the inner systems were reduced to a minimum. In this way a recording system was obtained which under varying pressure conditions had a natural frequency of 40 to 60 per second, and which gave records representing the volume changes in a short segment of the artery. It is obvious that the wave characteristics are likely to be more clearly demonstrated when this is the case, for, if a large length of artery contributes to the record, the wave characteristics must represent some integration of complicated waves occurring at different times in different segments of the artery.

Method. *Apparatus.* The *cuff* consists of an armlet of flexible leather controlled by three straps (see fig. 1). Within this is a large compressing

rubber bag of the ordinary type (21 × 12.5 cm.); within this again, covering the lower $\frac{2}{3}$ of the other bag, is another intermediate insulating rubber bag (14.5 × 8 cm.) separated from the other bag only by a thin cloth cover; in the innermost situation is a third recording rubber bag (10 × 4 cm.), the upper edge of which lies at the center of the compressing cuff. This third bag is separated from the second by a layer of cloth, and, if desired, also by a layer of lead foil paper. This last is cut into strips and mounted on cloth or adhesive tape, so that it opposes no resistance to bending; it may then be inserted without modifying appreciably the pressures applied, and

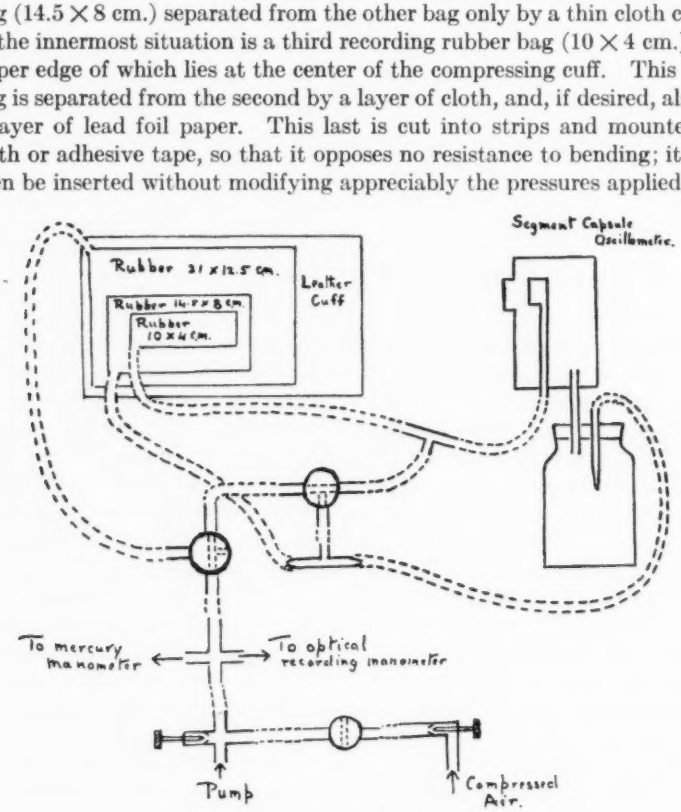

Fig. 1. Diagram of 3 bag system (see text)

its presence does appear to improve slightly the insulation of the third bag. However it is not essential and almost equally good records may be obtained without it. The cuff is applied to the arm, so that the third bag lies directly in contact with the skin over the artery. The cuff must be applied rather loosely, so that a moderate volume of air can be inserted into the two inner bags without unduly compressing the arm. In practice, if the volume of air contained is found to be too small, the cuff is slightly loosened, and the records are repeated.

The *recording system* consists of the segment capsule of 1 cm. diameter described in the previous paper, covered with light rubber dam, or, if used

for records on the dog's thigh (and probably also for a child's arm), with lighter condom rubber. The capsule is contained in an air-tight metal box with a glass window, also previously described, of 7 cm. height and 6 cm. diameter; a small concave mirror of 1 meter focal length is used for recording the pulsations of the capsule, and light from an incandescent 6 volt-100 watt ribbon lamp with an intervening slit is focussed on it. If desired an automobile head light lamp may be employed instead; but it is less well adapted for simultaneous records from several capsules. The innermost bag is connected by heavy rubber tubing through a T-tube to the segment capsule. The intermediate bag is connected to the containing box through a T-tube and an extra reservoir of 400 cc. capacity; in these connections are three capillary tube resistances which diminish the tendency of pulsations of the intermediate bag to affect the segment capsule. Air is introduced into these two systems either from a system of compressed air or from a pump through two glass 3-way stopcocks and the T-tube connections arranged as shown in figure 1. A needle valve allows air to be released if too much air has been introduced, and a second needle valve and a simple glass stopcock regulates the inflow of air, if a system of compressed air is employed. A four-way connection on the air supply connects the system also to two manometers, one a simple mercury manometer to allow the direct reading of the pressure, the second a recording manometer consisting simply of a segment capsule covered with heavy rubber of about 1 mm. thickness and also supplied with a concave mirror. With the system arranged as shown in figure 1 the recording system is distended to a pressure some 10 to 20 mm. Hg below the diastolic pressure; usually a pressure of 40 to 50 mm. is employed for the normal individual and one as high as 100 mm. or more for arterio-sclerotics with hypertension. The pressure is carefully adjusted to an exact level (e.g., 50 mm.), and a short record is then taken. This serves for standardisation and gives the position of the slit on the record at this pressure (e.g., 50 mm.); it indicates as well the initial pressure employed in the recording inner bags. The right hand stopcock (in fig. 1) is then completely closed by moving it through 45° anticlockwise to an intermediate diagonal position, leaving both sides of the segment capsule at a pressure of 50 mm. and completely closed and independent; the pulsations of the artery are transmitted to the segment capsule, and only to a negligible extent to its containing box. Both systems must be absolutely air-tight. The second 3-way stopcock, that on the left in figure 1, is then turned through 90° clockwise, so that air can be driven into the compressing unit.

The *compressing unit* consists of the larger outside bag connected through the second stopcock to the air inlet, whether this be a pump or compressed air, and by the intervening 4-way junction also with the mercury manometer and recording manometer. Air is introduced into it, until the pressure

reaches the same level as that in the recording system, when a record is started to show the wave changes during inflation, provided that steady inflation from a compressed air supply is possible. If this is not available, then the pressure is raised to some level well above systolic pressure, and records are taken during deflation only. As the pressure is raised in the compressing system, it is applied to the arm through the other two bags, the air in which is compressed to the same pressure. Yet the only movement of air that has to take place in the recording system is that necessitated by the equalisation of pressures between the bags and the segment capsule, its container, and the damping air reservoir. The only drift of the segment capsule that occurs is that occasioned by the resistance offered to attainment of equilibrium by the capillary resistances, and if these are properly chosen and if inflation and deflation are carried out slowly, this drift should be slight. When the compression has been carried well above the systolic pressure level, it is regulated exactly to some definite level (e.g., 150 mm.) and a short record is taken. This serves also as a standardisation point and indicates the level of the recording manometer image at a second pressure. Any intermediate pressure may be then read accurately enough by linear interpolation between these two points; each record contains its own standardisation. Deflation is carried out in a similar manner.

Drifting of the oscillograph record. A normal tendency to drift has already been described—drifting to a larger extent depends either on the presence of a leak, or on an inadequate total air content of the two inner bags. For it is obvious that, if the intermediate bag contains a small volume of air, the volume may be too small, when compressed to a higher pressure, to fill the box and air reservoir at the higher pressure, and under these conditions the intermediate bag may become completely collapsed. Then the whole balancing system ceases to function. The remedy is to loosen the cuff slightly and utilise a somewhat larger volume of air in the recording system. When serious drift occurs from whatever cause, the record can often be rendered usable by simply renewing the balance on the two sides of the segment capsule by turning the right-hand stopcock through 45° clockwise for a moment, thus putting the two sides of the capsule into connection with one another and allowing readjustment of the pressure levels.

In addition to the oscillometer proper it is of great assistance in the interpretation of records to have also a record of the pulsations of the artery below the cuff. An *arterial pulsation record* is readily obtained by utilising a glycerine capsule tambour on the brachial artery at the elbow connected to a simple segment capsule covered with light rubber dam or with condom rubber, and this system will readily also give, as will also the oscillometer, indications of such sounds that are produced that are of relatively low frequency. The main difficulty is that this record tends to

drift enormously owing to the swelling of the arm with venous congestion. Two alternative methods may be used to render this drift negligible; into the connecting tubing a T-tube may be inserted with a very small open side tube, which allows the escape of air in accordance with slow volume changes, while it interferes in only a minor degree with the more rapid pulse vibrations; or secondly, the air connections may be completely closed while the receiving tambour is applied with a steady pressure. This may be attained by slinging it over the arm by a weight, instead of applying it by a strap. The former of these methods is the simpler but gives a more or less distorted pulse curve; the latter is theoretically better but necessitates the fixation of the arm in an abducted position on an arm rest, and vibrations of the arm may still distort the curves. In fact in this method the movements of the whole arm which result from blood entering the arm at the axilla and also those resulting from respiratory movements usually give a good record of small amplitude of both pulse and respiratory rates at all pressures of the cuff. On the whole the latter method is preferable for standard use, the former for rapid adjustment in a portable system, or where the subject's freedom of movement should not be impaired.

If *lighting* of the segment capsules is attained by the use of a ribbon lamp at a distance of about 1 meter, all the mirrors may readily be arranged within the image of a single lamp and the difficulties of multiple lamps may be avoided. An additional capsule may also be added if desired and be connected to an open cup strapped to the abdominal wall: respiration can then also be recorded with certainty, and respiratory variations in pressure may be analysed.

The records obtained and the criteria used. Records obtained during the more familiar process of slow deflation as well as those with the less familiar slow inflation will be discussed and the curve characteristics at various pressure levels will be considered separately.

Pressures above end systolic pressure. At such pressures the oscillograph record, and also often that obtained from the artery at the elbow, still shows slight pulsations with each heart beat with the highly sensitive systems employed. That in the oscillograph presumably results from impulses from the uncompressed artery above the cuff transmitted to the innermost cuff, partly through the system of cuffs, but probably mainly through the muscle. The pulsations diminish slightly as the pressure is raised but are not obliterated. The pulsations apparently obtained from the artery at the elbow are small and readily recognised. They seem to depend on a vibration of the whole arm produced by blood passing into the arm through the curved circuit of the axillary artery.

End systolic pressure. The changes observed are similar whether deflation or inflation is employed. At a pressure level approximately corresponding to end systolic pressure, the pulse wave is able to pass one half

the width of the larger compressing bag and to influence directly the innermost recording bag. As this bag has its upper margin at the center of the compressing bag and at the point where the pressure is maximal, the attainment and passage of this point are significant. When this occurs, a definite new wave is superimposed on the simple wave seen at higher pressures, and as the pressure is dropped this new wave rapidly grows in importance until the other smaller wave is scarcely recognisable. This criterion occurs at cuff pressures definitely above those at which the pulse waves are able to

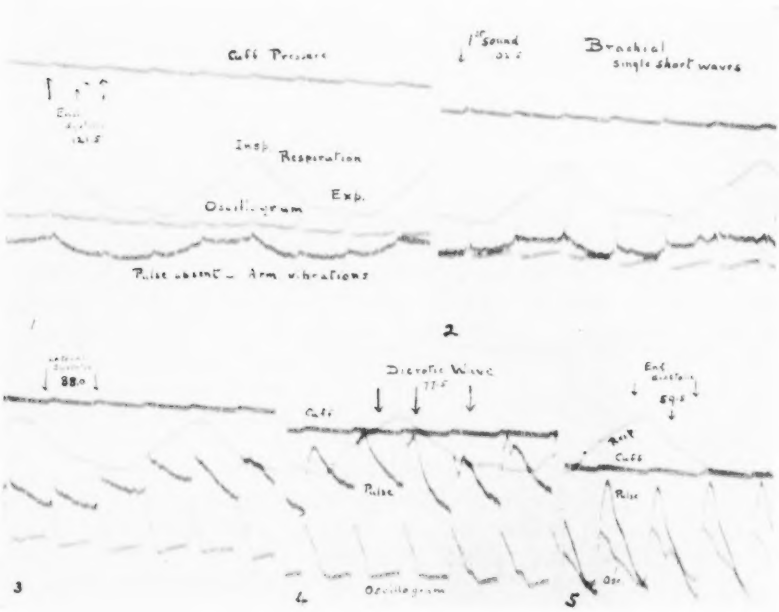

Fig. 2. Record of deflation on subject A, showing cuff pressure, oscillometer and brachial pulse records as well as a respiratory record (see text).

traverse the whole cuff and affect the tambour at the elbow. A good example may be seen in figure 2 from an experiment in which records were obtained from the brachial artery by means of a tambour suspended by a weight, and in which a respiratory record was also obtained. In the first section reproduced the pulse and respiratory movements may be read from the brachial record although a high pressure existed in the cuff; the first pulse of the oscillograph is a simple wave with some secondary components, at the second pulse a second sharp impulse appears which grows rapidly as the pressure in the cuff is allowed to fall and which shows varia-

tion in size with respiration; it tends to be maximal in the later stage of expiration.

In the second section, at a cuff pressure some 19 mm. lower, there is a change in the brachial record which indicates systolic pressure as measured by the brachial pulse. Upon the previous waves is superimposed another with a much sharper up-stroke which is accompanied by sound vibrations. This may be clearly seen starting in the fourth pulse of this section at a cuff pressure of about 100 mm. A similar wave may also be detected in the first pulse of this second section at a cuff pressure of 102.5 mm. (marked in figure as 1st sound), but it disappears again in the second and third pulsations during the inspiratory fall of pressure. Systolic pressure as indicated by a recordable brachial pulse with sounds would therefore be placed as 100 to 102.5 mm. according to the respiratory phase, a level intermediate between end systolic and lateral systolic as estimated by the oscillograph record. By auscultatory methods systolic pressure would probably have been estimated as 100 mm.

Lateral systolic pressure. As the cuff pressure is lowered the oscillograph record changes somewhat as in the third section of figure 2; the sharp descent of the main pulsation is broken by a slight shoulder which is definitely present in the third pulsation. At the same time the brachial pulse changes from a sharp upstroke with a gradual fall, on which sound vibrations are superimposed, to one in which the sharp upstroke is followed by a secondary rise, or plateau; this plateau is definitely seen in the sixth pulse of this section near the end of expiration when the blood pressure is highest; it is fairly definite in the fourth. The more prolonged pulsation with a second maximum presumably depends on a cuff pressure which is below that of the lateral pressure within the artery. According to Broemser (1928) the maximum velocity is reached earlier in the pulse cycle than the maximum lateral pressure, and consequently, if the pulse is obstructed, pressure development from conversion of kinetic into pressure energy should precede the maximum lateral pressure wave. The value for lateral systolic pressure so determined from the oscillograph record is very slightly higher than that estimated from the brachial record. This is to be anticipated, since the extreme peak of the wave might not last long enough to pass the whole length of the cuff. The value used is therefore that determined from the oscillograph, and the brachial record serves to confirm the selection of the point of change, which is often, as in this record, not very definite. During inflation the change from a cuff pressure below lateral systolic pressure to one above it usually produces a much more definite change in the oscillograph. An example from a subject with much slower respiration, sinus arrhythmia and marked respiratory variations in blood pressure is shown in figure 3; the first pulse of the third section gives an oscillograph with a plateau type of peak, the second has a very definite shoulder, the third a slight shoulder; the brachial pulse similarly loses its

secondary wave at about the same point. This would place lateral systolic pressure as 119 mm. at the end of expiration. In the 6th and 8th pulsations the oscillograph also shows some indication of apparent secondary waves which complicate the interpretation of the oscillograph record; these are probably artifacts produced perhaps by some vibration of the

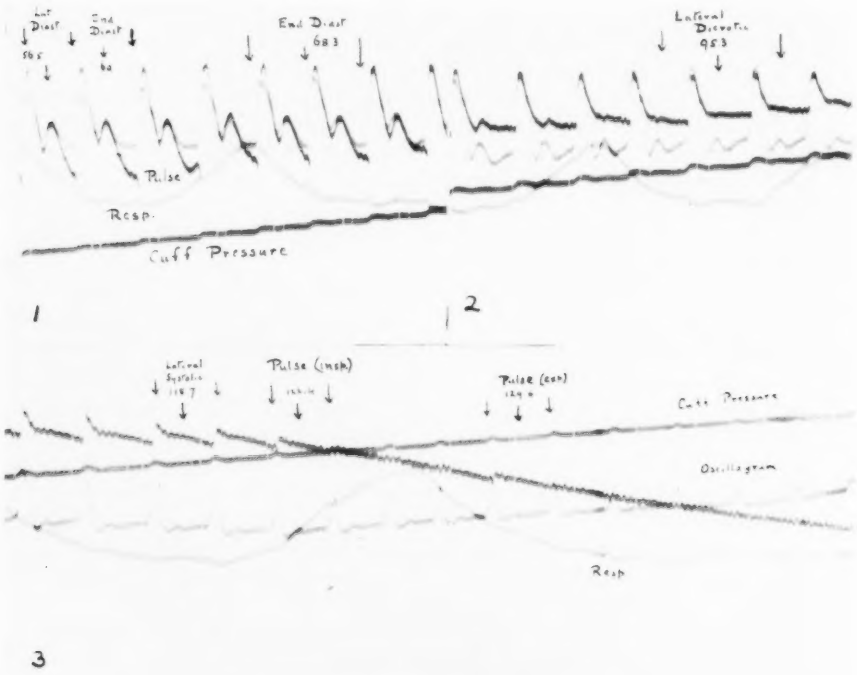

Fig. 3. Record of inflation on subject Su with slow irregular respiration and some sinus arrhythmia. The oscillometric criterion for end systolic pressure occurred at a still higher pressure and is not reproduced. The consistent presence of a brachial pulse at pressures below 122 mm. may be noted, with a disappearance of this pulse at this level in inspiration and return of the pulse in the middle of expiration even though the cuff pressure was then 129 mm. Systolic pressure on the basis of the brachial pulse could therefore be stated to vary with respiration between 122.4 and 129.6.

arm, since the brachial pulse indicates that the pulse is unable to pass the whole length of the cuff, and the cuff pressure must certainly have been above lateral systolic pressure.

End diastolic pressure. At moderate cuff pressures the oscillograph

record has a definite flat area during the latter part of the pulse cycle; this flat area presumably indicates a compressed vessel with an absence of pulsation; this is clearly seen in figure 2, section 4. In section 5 of the same figure a short flat area may still be recognised in the first two pulsations, but it completely disappears by the third pulsation, which shows the sharp angle of a normal pulse. A similar transition may be seen in figure 3 during inflation; the flattened type of curve in diastole appears in the second pulse of the first section; but the sharp angle of the normal type reappears in the 5th pulse to disappear again at the 6th. The 5th pulse occurs in the middle of expiration at the period of maximum pressure from respiratory variations, and at a time when the diastolic pressure is particularly raised by a speeding up of the pulse rate as a result of the sinus

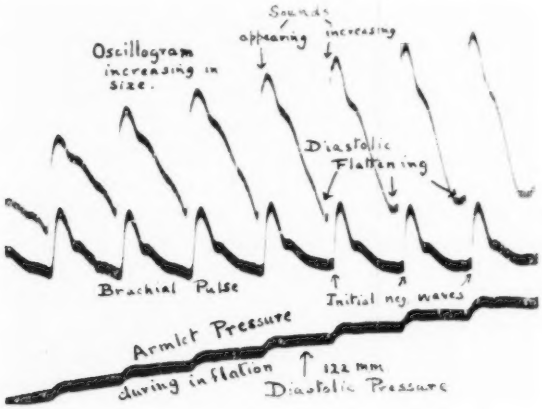

Fig. 4. Record from subject K with arteriosclerosis showing agreement of various diastolic criteria during relatively rapid inflation at about 4 mm. per second.

arrhythmia. The record therefore demonstrates an alteration of the end diastolic pressure with respiration with similar time relations and characteristics to those demonstrable in direct records in animals.

Lateral diastolic pressure. The pulsations from the brachial artery may be seen to be preceded at most cuff pressures by an initial sharp negative wave.[1] This may be clearly seen in figure 2 in section 5 where it diminishes to a slight notch by the last pulsation; a similar wave is seen in figure 3, section 1, except for the first pulsation which rises without any such initial negative wave. This negative wave appears as a rule very definitely and clearly at a pressure 2 to 14 mm. below that which gives the end diastolic

[1] This wave has also recently received attention from Frank and Wezler: Zeitsch. f. Biol., 1931, xci, 439.

pressure criterion. It therefore probably indicates lateral diastolic pressure, as has been previously suggested by Erlanger (1921) and by Bramwell and Hickson (1926).

The general agreement between the different criteria for diastolic pressure, when the effects of rapid deflation are avoided, may be seen in figure 4. This record in a subject with hypertension was obtained with somewhat rapid inflation, and under these circumstances the criteria of increasing sounds, rapidly increasing size of oscillations and development of an initial negative wave in the brachial pulse all appear together at approximately the same level as that which gives von Recklinghausen's (1930) criterion for end diastolic pressure.

Dicrotic wave pressure. At the higher cuff pressures there is no doubt that the dicrotic wave is unable to pass the cuff and is absent in both the oscillograph and brachial records; this is true for instance in the first three sections of figure 2. In the fourth section of this figure the dicrotic wave becomes apparent in the oscillograph in the third pulse as a small wave rising from the base line, and as a shoulder on the descending limb of the main curve in the fourth pulse. If the artery is patent over at least the main part of the cuff at the time of this fourth pulsation, the lateral pressure of the dicrotic wave should be greater than the cuff pressure at the fourth pulse. The end pressure of the dicrotic wave should be lower than the cuff pressure at the time of the first pulsation of this section[2] and possibly lower than that at the second, which is accompanied by an extremely small wave at the time of the dicrotic wave. The lateral dicrotic pressure may be taken as lying between these points but any exact distinction between end and lateral pressures does not appear possible, even provisionally, in records such as this where the dicrotic wave is throughout small. When the dicrotic wave is large, as in figure 3, the curves are different. It will be noticed that a dicrotic wave is evident in the oscillograph record at all cuff pressures up to that at which the pulse only just reaches the brachial tambour, at a pressure therefore which must be well above the true lateral dicrotic pressure. Such waves are probably transmitted through the muscle and cuffs from vessels more centrally situated. The dicrotic wave disappears in the brachial record in the 5th pulse of the second section; at about the same point the dicrotic wave in the oscillograph record changes from a flat plateau-topped curve to a simple peaked curve. This change is assumed to correspond with a cuff pressure rising above the lateral pressure of the dicrotic wave, but if this be true it is not possible to pick out any definite change at a somewhat higher pressure corresponding to the end pressure of the dicrotic wave. The pressure of the dicrotic wave may therefore be read with moderate accuracy, and a criterion for lateral pres-

[2] The occurrence of a wave on the oscillographic down stroke of this pulsation is considered an artifact, but it may indicate that at this stage of respiration the pressure of the dicrotic wave rose above that of the cuff.

sure may provisionally be selected but exact discrimination between end and lateral pressure for the dicrotic wave is in certain cases extremely difficult, and in all somewhat problematical.

Mean pressure. It will be clear from figures 2 and 3 that there is no very definite point at which the pulsations are largest, and, even if there were, it is doubtful (see paper I of this series) whether it would be a reliable index of mean pressure. The pulse curve obtained from an uncompressed artery must represent however the lateral pressure changes within the vessel, though it will be distorted to some extent by the variable distensibility of the vessel wall at different pressures. It seems best therefore to obtain an optical record of the brachial pulse (without distention of the cuff and with a completely closed system between the brachial tambour and recording segment capsule), and to regard this as a graphic record of the lateral pressure changes. If graphic integration of this curve be carried out (e.g., by cutting out the curve and weighing) the average height may readily be determined, and in this way mean pressure may be estimated. For instance the lateral systolic pressure was estimated on the average in the experiment illustrated by figure 2, as 89.0 mm. and the lateral diastolic pressure as 53.7 mm.; the height of the brachial record pulsation could therefore be considered as equivalent to the difference, or 35.3 mm. of mercury. The average height of the brachial record was 52 per cent of the maximum height, so that the mean lateral pressure was considered as equal to $53.7 + {}^{52}_{100} \cdot 35.3$ or 72 mm.

Probable accuracy. Mechanical factors. The factors concerned in accurate indirect estimates of blood pressure with single and double bag systems discussed in the first paper of this series would appear to be equally significant in the present system. There is at any rate no evidence to indicate that the interposition of the other bags interferes with the transmission of pressure to the limb, provided that any insulating material used offers no appreciable resistance; for, if the middle and outer bags be connected simultaneously to two manometers, the pressures read during compression do not differ materially.

The *criteria employed* are open to criticism, since their selection depends on an arbitrary application of theoretical principles. They are based, it is true, on von Recklinghausen's very interesting analysis of the wave forms, but the actual records obtained by this system for a short length of artery differ in many points from those obtained from a longer section by von Recklinghausen. Doctor von Recklinghausen has very kindly examined a few of the records; he does not always agree with the interpretations here set forth, though his selection of points of change has little effect on the estimations of the pressures. In particular he tends to read diastolic pressure at a somewhat lower level on the basis of a change of slope in curves with a sharp angular depression. This we do not consider warranted if the oscillographic change is used to indicate end diastolic pressure.

The agreement between the various diastolic criteria with relatively rapid inflation such as that used in figure 4 raises the question whether the fine distinction here drawn between end and lateral diastolic pressures is really warranted or whether it depends on some artifact. That it is a real phenomenon is however indicated by the effect of meals; table 1 shows the changes observed on 5 occasions in 4 subjects; records were obtained after a long period without food and again 15 to 60 minutes after a meal. It may be noted that in two subjects there was an increased difference between estimated end and lateral systolic pressures after the meal, in the other three experiments this difference was reduced; in all but one experiment the difference between end and lateral diastolic pressures was increased. The differences appear therefore to be dependent on some physiological factor and not to be instrumental in origin; if they are indeed artificial, then the error must vary with the character of the pulse wave. Additional evidence that the records provide a measure of actual differences is the fact that the

TABLE 1

SUBJECT	BEFORE MEAL								AFTER MEAL							
	Pulse rate	Pulse wave velocity	End systolic	Lateral systolic	Difference	End diastolic	Lateral diastolic	Difference	Pulse rate	Pulse wave velocity	End systolic	Lateral systolic	Difference	End diastolic	Lateral diastolic	Difference
M	74.3	3.02	110.0	84.2	25.8	55.5	52.3	3.2	77.3	3.64	115.0	96.2	18.8	78.0	64.0	14.0
St	72.5	4.01	128.1	107.7	20.4	76.1	68.3	7.8	81.2	4.88	136.5	114.0	22.5	78.6	71.7	6.9
Su	69.4	3.51	129.4	101.0	28.4	63.1	58.8	4.3	72.6	3.51	127.0	101.9	25.1	61.9	55.3	6.6
Su	66.1	4.30	143.4	113.6	29.8	65.0	60.6	4.4	80.1	5.86	150.3	126.1	24.2	61.7	54.0	7.7
K	62.0	5.89	196.0	171.0	25.0	103.0	100.5	2.5	71.4	7.00	216.0	186.0	30.0	117.5	114.0	3.5
Means	68.8	4.14	141.4	115.5	25.9	72.5	68.1	4.4	76.5	4.98	149.0	124.8	24.2	79.5	71.8	7.7

differences between end and lateral pressures (both systolic and diastolic), are of the same order of magnitude and show the same degree of variation as those observed in the dog's thigh by direct measurement (Bazett and Laplace, paper I).

Another method of checking the accuracy of the estimates is to compare the apparent position of the dicrotic wave in the pulse wave as determined by the oscillograph measurements with its position on a photographic pulse curve. Thus for instance in the experiment illustrated by figure 2 the mean estimate of lateral systolic pressure was 89.0 mm., that of lateral diastolic 53.7 mm.; the dicrotic wave was estimated to have a pressure of 77.3 mm. The dicrotic wave according to this estimate occurred when the pressure had fallen 33.2 per cent toward the diastolic level; on a pulse tracing it appeared to occur after the pressure had fallen 39 per cent toward the diastolic level. Considering the possible errors in both estimates, the agreement is fairly good. A comparison of this type between direct esti-

mates of the dicrotic wave pressure, and estimates made from the pulse curve on the assumption that the lateral systolic and diastolic pressures are correctly measured is shown in table 2. Cases have been selected which show the best and poorest correlation, so that the figures give a fair representation of the sort of agreement obtained; as far as possible records also utilised for other tables have been employed. It must be remembered however that the measurement of lateral pressure of the dicrotic wave is probably the least accurate of the measurements made; if there is a con-

TABLE 2

Comparison of dicrotic wave pressure as estimated by oscillograph record and as calculated from pulse curve from oscillographic estimates of lateral systolic and diastolic pressures

SUBJECT	CONDITION	PULSE RATE	LATERAL SYSTOLIC	LATERAL DIASTOLIC	PULSE ESTIMATE OF DICROTIC	OSCILLOGRAPH ESTIMATE OF DICROTIC	DIFFERENCE	COMMENTS
1. M	Basal	74.3	84.2	52.3	67.0	69.3	+2.3	See table 1
1a.	After meal	77.3	96.2	64.0	84.5	89.7	+5.2	See table 1
2. St	Basal	72.5	107.7	68.3	90.7	94.2	+3.5	See table 1
2a.	After meal	81.2	114.0	71.7	93.3	96.0	+2.7	See table 1
3. Su	Fasting	69.4	101.0	58.8	88.2	80.7	−7.5	See table 1
3a.	After meal	72.6	101.9	55.3	85.3	82.6	−2.7	See table 1
4. Su	Fasting	66.1	113.6	60.6	90.8	94.9	+4.1	See table 1
4a.	After meal	80.1	126.1	54.0	88.4	85.2	−3.2	See table 1
5. Su	Basal	56.9	117.7	80.5	103.4	105.3	+1.9	See table 3
6. K	Resting	62.0	171.0	100.5	155.5	153.0	−2.5	See table 1
6a.	After meal	71.4	186.0	114.0	170.0	166.5	−3.5	See table 1. Arterio-
7. K	Basal	63.8	179.8	110.6	145.2	147.4	+2.2	sclerosis
8. A	Resting	78.0	89.0	53.7	75.2	77.3	+2.3	See fig. 2
9. A	Sitting	91.5	108.9	69.5	89.2	91.2	+2.0	See table 3
10. G	Basal	60.0	103.0	65.0	88.0	87.0	−1.0	
11. Ste	Resting	67.5	119.5	76.0	91.1	102.5	+11.4	
Mean values.....		71.4	120.1	71.8	100.3	101.4	±3.6	

siderable disagreement between the two estimates for dicrotic pressure, no reliance can be placed on either, but the method need not be condemned entirely on this account. To some extent at any rate such discrepancies may be due to variations in the character of the pulse curve with respiration. For instance in subject Su with a slow respiration and sinus arrhythmia the dicrotic wave varied in the pulse record (table 2, no. 2) during a single respiratory cycle from a value of 21 per cent towards the diastolic level to 37.8 per cent. Discrepancies may therefore sometimes depend on inadequate sampling and on a consequent inaccurate estimate of the mean value.

TABLE 3

Consistency of readings

RECORD NUMBER	INFLATION OR DEFLATION	PULSE RATE	END SYSTOLIC	LATERAL SYSTOLIC	DICROTIC (LATERAL)	END DIASTOLIC	LATERAL DIASTOLIC	COMMENTS
				Subject A				
1	I	93.5	115	104.5	82.5	70		Sitting, resting for about 15 minutes before records taken
2	D	94	117.5	102.5	83	66.5	64	
3	I	90	120	106	82.5	66.5	59.5	Records 1, 2, 5 and 6 obtained with one armlet. Records 3, 4, 7 and 8 with another of slightly different size and with slightly different covering
4	D	95	116.5	105	86.5	70	64.5	
5	I	91	129	113	99	80	72.5	
6	D	93.5	133	113	96.5	78.5	72	
7	I	87	129	116	101	81	76	
8	D	88	129	111.5	99	81.5	78.5	
Means:								
All readings....		91.5	123.5	108.9	91.2	74.9	69.5	
All inflations....		90.4	123.2	109.9	91.2	75.6	69.3	
All deflations...		92.6	123.8	108.0	91.2	74.1	69.7	
				Subject Su				
1	I	58.6	145.3	117.1	100.4	80.7	75.7	Basal conditions. Resp. rate steady 7.0 per minute. Sinus arrhythmia
2	D	59.8	146.0 135.0	112.4	99.3	77.3	75.0	Resp. rate varying between 5.6 and 15.6 per minute
3	I	57.7	145.0	114.7	101.0 108.0	85.4 89.3	82.2 85.0	Resp. rate varying between 7.4 and 14.9 per minute
4	D	56.3	141.0 139.5 137.0	120.0 118.0 115.0 108.0	112.0	84.0	82.2 77.0	Resp. rate varying between 5.8 and 14.6 per minute
5	I	53.9		119.4 122.0	109.3	89.4	84.7	Resp. rate 7.4 per minute
6	D	56.6	139.5	110.0	102.0	82.7	81.0	Resp. rate 14.3 per minute
7	I	56.2	143.0	124.6	108.6	81.2	77.3	Resp. rate 16.1 per minute
8	D	56.0	147.5	127.2 122.0	107.2	90.0 87.3	84.7	Resp. rate varying between 10.8 and 15.9 per minute
Means:								
All values.....		56.9	141.8	117.7	105.3	84.7	80.5	
Inflations.....		56.6	144.4	119.6	105.5	85.2	81.0	
Deflations.....		57.2	140.7	116.6	105.1	84.3	80.0	

Consistency of the measurements. There is considerable evidence that respiratory variations, which are hardly detectable by ordinary indirect methods, are readily demonstrable by this method and account for many

of the apparent inconsistencies observed. It is at any rate clear that if inflation and deflation are carried out slowly, no appreciable difference is observed between the two sets of values. In table 3 individual estimates in 2 subjects from 8 records each are detailed; one half of the records were obtained during inflation, the other half with deflation. Subject A had a relatively normal respiratory rate with evidence of only slight respiratory variations, while subject Su showed a very slow respiratory rate with irregular periods of faster respiration, and a definite respiratory variation both of pulse rate and blood pressure. It is clear that in both cases the mean values obtained with inflation and deflation are practically identical, and yet individual values vary considerably—over a range of 10 to 19 mm. in both subjects. In subject A the variations appear to be due mainly to a gradual change in the subject with rest (note change in pulse rate) with only minor variations, not exceeding 5 mm. between consecutive records. In subject Su on the other hand an ample rest (50 min.) had been allowed before records were commenced and there is little or no evidence of any progressive change. With very slow inflation or deflation the criteria might occur, disappear and reappear at different pressure levels in the same record according to the respiratory phase. Consequently some of these data are plotted in figure 5 according to the respiratory phase at which the criteria were observed. For convenience a long respiratory cycle has also been drawn but data are inserted according to the stage of respiration rather than time. It will be observed that the major differences are definitely related to respiration, and that the scattering is not great in view of the facts that the measurements were made over a period of one hour, and that the respiratory rate itself was not uniform. Some values for systolic pressure obtained on the same subject on another occasion in a single record with very slow deflation show the same type of curve and are also plotted in figure 5. In all records the size of pulsation indicated a maximum systolic pressure early in expiration. It is worth noting that the character and magnitude of the respiratory variations are quite similar to those observed in direct records from animals with sinus arrhythmia.

In most subjects reliable estimates of pressures may be obtained by making 4 records and taking the mean of all estimates; when however respiratory variations are as great as in the case cited, the respiratory movements should be recorded and mean values should be estimated approximately after due consideration of the respiratory phase in which the criteria appeared and the probable character of the respiratory waves, unless a larger number of values are available.

It is obvious that the accuracy of the method should be more adequately checked by experiments on dogs of the type of those described in the first paper of this series. A few experiments of this character have been performed and the method appears to have as great an accuracy for end sys-

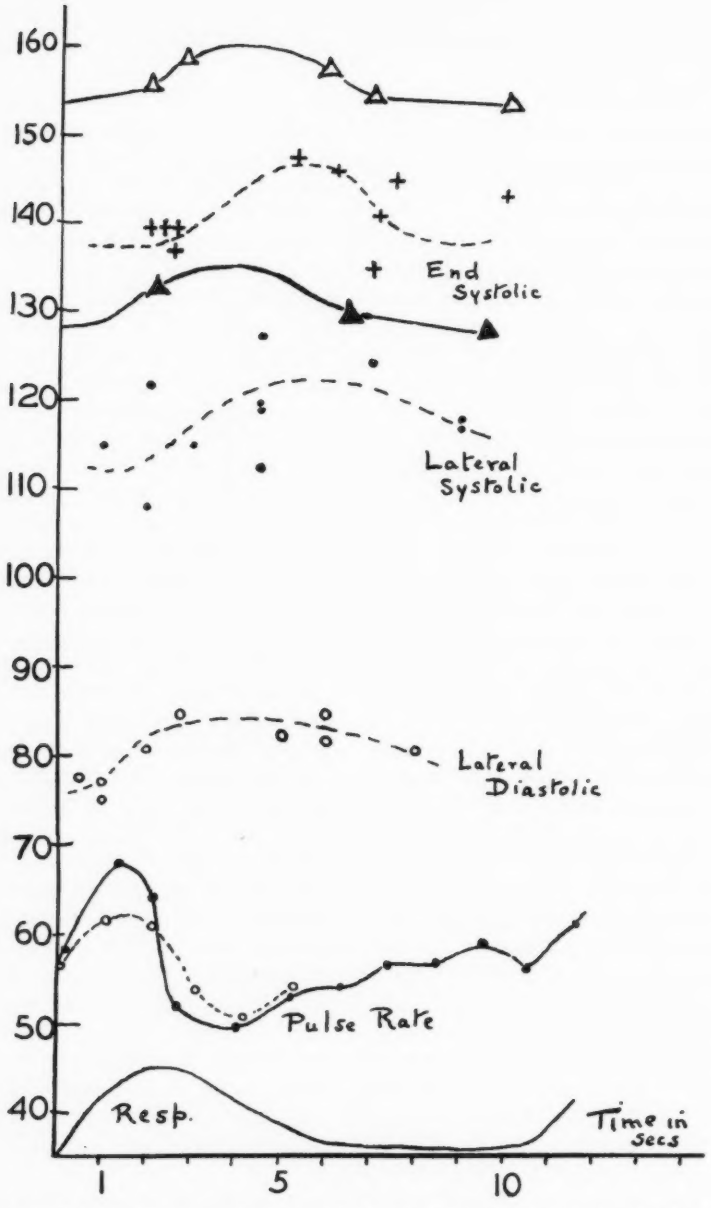

Fig. 5. Values of pressures in subject Su (table 3) plotted in relation to the respiratory phase with two examples of the respiratory variation in pulse rate. End systolic values + - - - +; lateral systolic values ● - - - - ● Values for end and lateral systolic pressure from a single record with very slow deflation in the same subject on another occasion (table 2, 4 a) are also plotted; end systolic △——△; lateral systolic ▲——▲.

tolic and end diastolic pressures as was described earlier for the von Recklinghausen criteria. Further progress in such checks, however, is for the present hindered by the absence of adequate criteria for the other points in records from the dog's thigh.

It was suggested to us by Professor Erlanger that if the innermost bag was used at the lower margin of the cuff instead of at its center, the initial negative wave, often demonstrable in the oscillograph record would be rendered more definite, so that it could be used as the criterion of lateral diastolic pressure and the extra complication of a brachial record might be avoided. This change has been attempted, but so far the smaller bag has not apparently been removed far enough from the compressing area to demonstrate this criterion clearly in all records, but the possibility of so simplifying the apparatus should be borne in mind.

We would like to take this opportunity of thanking Doctor von Recklinghausen for his interest in this work and for critical comments which we have received by mail, as well as to Professor Erlanger for his suggestions.

CONCLUSION

A method of obtaining oscillographic records and of estimating blood pressure from them is described. By the use of three air bags of different size one within the other a system of high natural frequency may be obtained, and records obtained with little distortion during continuous slow inflation or deflation. Criteria are described and interpreted as modifications of those detailed by von Recklinghausen.

The method appears to indicate both end and lateral pressures for systole and diastole, as well as the pressure of the dicrotic wave. Data are presented which demonstrate an agreement between the estimates for dicrotic pressure so reached and those made from the character of the pulse wave and the estimated levels for lateral systolic and diastolic pressures. Figures are given which demonstrate that respiratory variations in blood pressure, both in systole and diastole, can be readily demonstrated, and approximately measured by this method.

The presence of respiratory variations in blood pressure can also be demonstrated with optical methods in records from the brachial artery below the compressing cuff. During deflation the first brachial pulse is recorded at a cuff pressure intermediate between the pressures that in the oscillograph give the end and lateral systolic criteria.

BIBLIOGRAPHY

BAZETT AND LAPLACE. 1932. This Journal, ciii, 48.
BRAMWELL AND HICKSON. 1926. Heart, xiii, 129.
BROEMSER. 1928. Zeitschr. f. Biol., lxxxvii, 264.
ERLANGER. 1921. This Journal, lv, 84.
VON RECKLINGHAUSEN. 1930. Zeitschr. f. klin. Med., cxiii, 1.

THE EFFECT OF OVERDOSES OF IRRADIATED ERGOSTEROL, ADMINISTERED FOR APPROXIMATELY TWO MONTHS, ON THE COMPOSITION AND STRUCTURE OF THE BONES OF RATS[1]

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In an early paper on the toxicity of large doses of irradiated ergosterol, Kreitmair and Hintzelmann (1928) described softening of bones in some of their experimental animals which had shown a chronic toxemia of long duration. Since then numerous investigators have reported decalcification of bone resulting from the administration of excessive quantities of irradiated ergosterol. In many of these studies, sufficient amounts of the vitamin D preparation were given to produce death within a period of approximately two weeks.

In a previous publication from this laboratory (Jones and Robson, 1931) objection was raised to some of this work on the basis that there had been no distinction made between the lack of normal calcification on the part of a growing animal and the actual withdrawal of calcium salts from the bones. In a series of experiments practically no evidence was obtained to show that any decalcification of bones of either growing or adult rats resulted from acute intoxication induced by irradiated ergosterol. Wiskott (1930) has also stated that acute poisoning with activated ergosterol produced no demineralization of the skeleton. However, with one adult rat which had received smaller doses for a long period of time, he noted a marked diminution in the ash of the bones. As stated in our previous publication, it was not presumed that it is absolutely impossible to bring about a decrease in the ash content of the bones by administering large doses of irradiated ergosterol. It was pointed out that although there might be a loss of a few milligrams per animal per day, as shown by Watchorn (1930) it could not be detected by chemical analysis of the bone in experiments the duration of which was two weeks or less. By slightly

[1] A report of this work was given before the Physiological Society of Philadelphia on December 14, 1931 (Jones and Robson, 1932. Amer. Journ. Med. Sci., clxxxiii, 299) and given as a demonstration at the Philadelphia meeting of the Federation of American Societies for Experimental Biology April 27 to 30, 1932.

decreasing the amount of vitamin D preparation, the time of the experiments could be increased and the animals would experience a chronic instead of an acute intoxication. It was thought that if an actual withdrawal of calcium salts from the bones occurred over a prolonged period of time, the loss would be of sufficient magnitude to be detected by ash determinations and by histological study of the bones. The data presented here deal with such experiments.

EXPERIMENTAL. Rats of different ages were used as experimental animals. By employing a diet low in calcium it was possible to increase the amount of irradiated ergosterol as it has been repeatedly shown that the toxicity of this drug is dependent to a certain extent upon the intake of calcium. In the first experiment 26 rats varying in age from 68 to 138 days were placed on a simple calcium-low diet consisting of 99 parts of whole yellow corn and 1 part of sodium chloride. After subsisting on the basal diet for about a week the animals were divided into 3 groups. As nearly as possible the animals were divided to give an equal distribution of sex and litter-mates in the three lots. The rats in the first group were killed, those in the second were continued on the same diet, while the animals of the third group in addition to the basal diet were given daily by mouth irradiated ergosterol solution directly from a tuberculin syringe. Several of the experimental animals died of intoxication; the remaining animals of group III and those of the second control group were killed within approximately 7 to 9 weeks after the beginning of ergosterol feeding. The right femur of each animal was removed for ash analysis, and the distal end of the left femur and the proximal ends of the corresponding tibia and fibula were removed and preserved for histological examination. Before extracting with alcohol and ashing, the right femora from all animals were x-rayed together on one film. Several exposures were necessary before the proper contrast in the negative was obtained. The bones were separated by strips of lead to prevent the deflection of the rays; this produced a sharper outline of the shadows.

In table 1 are given the detailed results of 6 animals of the same litter which are representative of this experiment. The averages for all animals of each group are also given. The heading of "diet" indicates to which group the animals belong and the amount of irradiated ergosterol given the experimental animals. The daily dose of vitamin D is expressed in Steenbock units accepting the standardization of the manufacturers as a basis for calculation.[2] The length of time that each animal was given the irradiated ergosterol is included in the table as this is of importance in the interpretation of the final results. The amount of ash is expressed in

[2] The irradiated ergosterol used was kindly furnished by Mead Johnson and Company, Evansville, Ind., and according to them had an approximate antirachitic value of 10,000X or 30,000D.

milligrams and in the percentage of the alcoholic-extracted dry femur. The density of the roentgenograms was determined by the use of a comparative densitometer.[3] Several determinations were made within corresponding areas of each bone and the average of each series was considered a measure of the density of any given bone. The results given in table 1 are expressed in absolute density units as described in the International Critical Tables.[4] Since the densities given are for the negatives of the roentgenogram, the bone densities are inversely proportional to the values given in the table.

TABLE 1

The effect of large doses of irradiated ergosterol on the ash content and densities of the femora of rats of various ages on a diet low in calcium

RAT NUMBER	SEX	DIET	TIME ON DIET	ASH		DENSITY
				mgm.	*per cent*	
1	M.	1st G*	0	211.8	60.3	0.23
2	F.	1st G	0	147.6	59.3	0.24
3	F.	2nd G†	52	165.2	61.6	0.24
4	M.	2nd G	56	203.8	61.3	0.24
5	F.	30,000‡	56	86.8	47.2	0.27
6	F.	30,000	52	158.8	53.0	0.28
Average all rats						
NUMBER OF RATS						
9		1st G	0	171.6	58.8	0.25
8		2nd G	58	173.9	59.2	0.26
9		3rd G**	48	128.7	51.7	0.29

* 1st G—Animals of 1st group (killed at beginning of experiment).

† 2nd G—Animals of 2nd group (continued on calcium-low diet).

** 3rd G—Animals of 3rd group (continued on calcium-low diet + 30,000 units of irradiated ergosterol).

‡ Daily dose of irradiated ergosterol expressed in Steenbock units.

As seen from the data presented, there is a decrease in the percentage ash in the bones of the animals receiving irradiated ergosterol as compared with either the first controls, those killed at the beginning of the experiment, or the second controls which were continued on the calcium-low diet throughout the experimental period. There were corresponding differ-

[3] The authors take pleasure in thanking Dr. I. S. Ravdin of the Department of Research Surgery for the roentgenograms and Prof. Charles Weyl and Mr. Reid Warren of the Moore School of Electrical Engineering for doing the densitometer determinations.

[4] International Critical Tables, vol. 5, p. 441.

ences in the densities of the bones as shown by the negatives of the roentgenograms. There were sufficient differences in the negatives to be detected by macroscopic examination, but by using the densitometer it was possible to obtain an exact measurement of these differences. There were certain exceptions to the general tendency of the animals receiving irradiated ergosterol to show a lowered ash content of the femora. Two of the experimental animals showed but a slight decrease in ash as compared to litter-mate controls. These two animals, however, died in 15 and 19 days respectively after the beginning of irradiated ergosterol administration. These results are in complete accord with our earlier observations which indicated that animals acutely poisoned with irradiated ergosterol experience no appreciable loss of bone ash. All experimental animals which received the vitamin D preparation for several weeks before death showed a noticeable decrease in absolute amount and percentage ash and in the density of the bones as compared with litter-mates of either control group.

The bones saved for histological study were fixed in neutral 10 per cent formaldehyde. After 48 hours' decalcification in 5 per cent nitric acid they were embedded in paraffin and sagittal sections cut, passing as nearly as possible through the centers of the tibiae and femora. These were stained with hematoxylin and eosin. The 9 animals in group I all had normal bones. In group II there were 8 animals, the bones of which were practically normal and as a group could not be distinguished from the bones of the animals in group I. It will be noted that ash content and density of bones of the animals in these two groups were also practically the same.

There were 9 animals in group III. Of these 9 animals the bones of 6 showed very marked changes. These changes were most marked in the cortical bone of the diaphyses which in the control animals was composed of compact bone of uniform thickness (fig. 1). In these experimental animals the cortical bone was extremely porous showing deep indentations and fenestrations filled with marrow (fatty and hematopoietic tissue, figs. 2 and 3). In most sections osteoclasts were very numerous. The inner surface of the bone was generally covered by a thin layer of osteoid tissue with numerous osteoclasts. The epiphyseal cartilages were unchanged. The spongy bone of the metaphyses showed irregular bony spicules with areas of resorption similar to the condition present in the cortical bone. The appearance was that of an osteoclastic resorption of bone with an accompanying new bone formation. In none of the sections had there been any filling of the defects with fibrous tissue. Where bone had disappeared the defect was filled with marrow. The animals showing this extreme resorption of bone all died from the irradiated ergosterol intoxication in periods varying from 52 to 63 days. All showed a definite reduction in the ash content and a decrease in the density of their bones.

Of the 3 remaining animals in group III one showed moderate and two

slight changes identical with the severe changes just described. The first of the 3 was killed at the end of 48 days. It showed reduction in ash content and decrease in density, but not as marked as in most of the experimental animals. It was the only one which was killed instead of being continued on experiment until it died of intoxication. The other two died in 15 and 19 days respectively; the former showed a slight decrease in ash content and density and the latter no change in these. In both the histological changes (fig. 4) were insignificant when compared to the extensive resorption of bone which occurred in the animals dying at a later date.

The histological structure of the bones of these rats gives definite evidence that the decrease in ash content and density is due to an actual loss of bone matrix. It also gives definite evidence that marked resorption occurs only when the intoxication is a prolonged one, since the rats dying in 3 weeks or less showed insignificant changes.

The lesion produced in the bones in these rats by irradiated ergosterol intoxication histologically is an extensive osteoclastic resorption especially of the cortical bone of the diaphyses. The lesions studied and described are those of the end stage of the process; a better understanding of their evolution could be obtained by sacrificing animals at intervals in order to observe the development of the lesions.

From the above data it is obvious that large doses of irradiated ergosterol can produce a marked destruction of bone. The process, however, does not seem to be a simple halisteresis, that is, a chemical removal of the calcium salts, but instead is a destruction and removal of the complete

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Fig. 1. A sagittal section through the diaphysis of the tibia of a rat which was killed at the age of 136 days as a normal control. The uniformly compact type of normal cortical bone is seen.

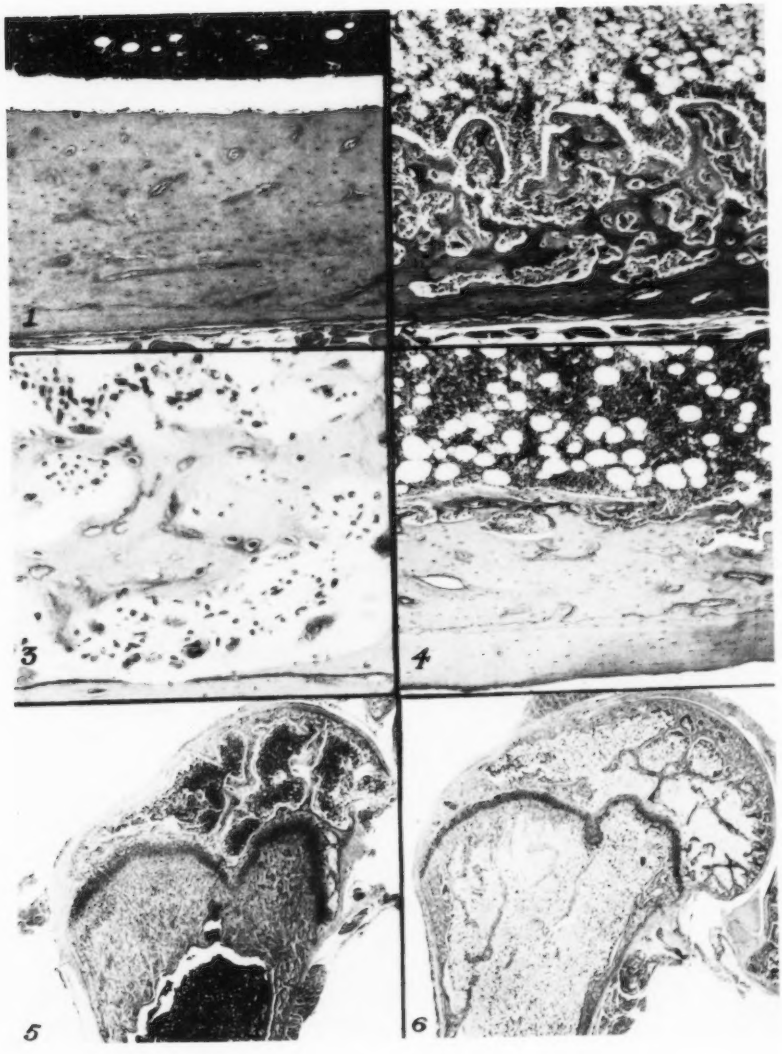
Fig. 2. A sagittal section through the diaphysis of the tibia of a rat which was placed on a low calcium diet plus 30,000 units of irradiated ergosterol at the age of 136 days. The rat died after 56 days on the diet. The extensive resorption of the cortical bone is shown. Osteoclasts are numerous.

Fig. 3. Higher magnification of the bone lesions seen in figure 2 showing numerous large osteoclasts.

Fig. 4. A sagittal section through the diaphysis of the tibia of a rat which was placed on a low calcium diet plus 30,000 units irradiated ergosterol at the age of 138 days and died after 19 days on this diet. The inner surface of the cortical bone shows several areas of resorption, but these are insignificant when compared to the extreme changes seen in figure 2.

Fig. 5. A sagittal section through the proximal end of the tibia of a rat which was placed on a low calcium diet at the age of 28 days and was killed at the age of 115 days. The marked proliferation of osteoid tissue in the metaphysis is well shown.

Fig. 6. A sagittal section through the proximal end of the tibia of a rat which was placed on a low calcium diet plus 3900 units of irradiated ergosterol at the age of 28 days and killed at the age of 115 days. The lack of osteoid tissue in the metaphysis is in marked contrast with the condition shown in figure 5.



Figs. 1-6

bone matrix, including organic as well as inorganic material. This destruction apparently is brought about by osteoclasts. It appears that the action begins at the inner surface of the cortex of the shaft and continues outward. In the bones of the animals which died within a short time after the beginning of irradiated ergosterol administration, the process was in its early stages as shown by the small indentations along the inner surface of the cortex.

The absolute amount of ash of the bones which showed an advanced porosity was probably less than that indicated by the percentage composition due to the simultaneous loss of organic material. In fact, it is possible that the observed decrease in the relative amount of ash was not caused by a greater removal of inorganic than organic constituents but was due to the subsequent formation of osteoid tissue and the penetration of the marrow into the recesses formed in the cortex. The marrow which thus penetrated into the cortex, however, was undoubtedly largely removed during the extraction with alcohol. The ash of the bones of some of the older animals which had received irradiated ergosterol decreased from a normal of about 61 per cent to approximately 50 per cent. At the time of their removal they were much more fragile than bones from young animals normally containing 50 per cent ash. This difference is probably due to the simultaneous loss of organic matrix on the part of the animals receiving irradiated ergosterol.

The picture presented by these bones is somewhat similar to that obtained by Jaffe, Bodansky and Blair (1931) in guinea pigs after administration of large doses of parathyroid extract. This raises the question of the interrelationship in the action of these two drugs. Because of the similarity of the symptoms produced by the giving of large doses of parathyroid extract and irradiated ergosterol, Taylor and associates (1931a, 1931b) are of the opinion that the toxicity of vitamin D overdosage is referable to its ability to stimulate the parathyroid glands. The results described here are another point in favor of their argument, but there are some definite differences between the bone picture as observed by Jaffe and coworkers and that obtained by us. One of the outstanding features of parathormone overdosage is the large amount of fibrous tissue which forms in the bone lesions, but which was absent in the bone lesions produced by irradiated ergosterol. In addition osteoid tissue was invariably present in the bones of the animals receiving irradiated ergosterol but not always evident in the animals to which parathyroid extract was administered. The results of further investigation on the interrelationship of these two substances will be reported later.

In a second experiment 37 rats of approximately 30 days of age were placed on either one of 2 basal diets. Diet 1 was the same as the basal ration used above and diet 2 was similar except 20 parts of the yellow corn

were replaced by an equal quantity of wheat gluten. As in the first experiment the animals were divided into 3 groups. After subsisting on the basal diet for about a week the rats of the first group were killed, those of the second were continued on the same diet, and the animals of the third group in addition to the basal diet were given by mouth irradiated ergosterol solution directly from a tuberculin syringe. Eight animals were given 3900 Steenbock units daily, 6 were given 5850 units and 3 received 7800 units. The experiment was continued for a period of about three months during which time none of the animals, with the exception of one, showed any of the usual external signs of irradiated ergosterol intoxication. Except for this one animal no evidence of any demineralization was obtained from these experiments, but as the amounts of vitamin D preparation administered were insufficient to produce toxic symptoms the results are inconclusive in this respect. However, several interesting points developed

TABLE 2

Effect of large doses of irradiated ergosterol, administered for three months, on the ash content of the femora of young rats on a diet low in calcium

(Results given are the averages for all animals in each group.)

GROUP NUMBER*	NUMBER OF RATS	INITIAL WEIGHT	FINAL WEIGHT	FEMUR	ASH	
		grams	*grams*	*mgm.*	*mgm.*	*per cent*
1	10	52.1		74.6	30.9	41.3
2	10	52.8	76.1	105.3	35.4	33.3
3	17	54.8	101.7	89.1	34.0	38.0

* Group 1—Animals killed at beginning of experiment.

Group 2—Animals continued on calcium-low diet.

Group 3—Animals continued on calcium-low diet + irradiated ergosterol in doses ranging from 3900 to 7800 Steenbock units daily.

which make the results worth recording. As the experiment progressed it was noted that the animals which received the irradiated ergosterol invariably developed a shambling gait, and some of the animals experienced considerable difficulty in locomotion before the experiment was terminated. At the same time these animals apparently were in considerable pain, for they squealed frequently when handled and also attempted to bite but seemed too weak to draw blood from the hands of the caretaker. Toward the end of the experiment the animals which did not receive the vitamin D supplement also showed some of these characteristics, but at no time were they as pronounced among the controls as among the experimental animals. At the end of the experiment all animals, except the one which had previously died, were chloroformed. A composite sample of blood was taken from a group of three of the control rats, as well as a sample from five rats receiving daily 3900 units of irradiated ergosterol and also from

five rats receiving 5850 units of the vitamin D preparation. The sera of these blood samples were analyzed for calcium and phosphorus. In addition to the blood sample the same tissues as mentioned above were removed and studied in a similar manner. The results of this experiment are summarized in table 2 which shows the average percentage of ash for each group, calculated from the weights of the alcohol-extracted bones, and also the average for the initial and final weights of the animals, the weights of the lipoid-free femora and of the ash obtained from the femora.

The results are divided into three groups corresponding to the three different types of treatment, viz., the first control group, that is the animals killed at the beginning of the experimental period, the second control group or the animals which were continued on the calcium-low diet, and the third or experimental group which received irradiated ergosterol. It can be seen that during the experimental period there was a decrease in percentage ash in the bones of both the second controls and the experimental animals as compared to the first controls. The bones of the animals not receiving vitamin D (group II) show a greater decrease in relative amounts of ash than those which were given the vitamin preparation. On further study of the data, it is observed that the decrease is not due to reduction in the actual amount of ash, as the average weights of ash from the bones of the animals of the three groups are very nearly the same. From the weights of the femora it can be seen that the relative decrease in ash is due to an increase of the organic portion of the bone. This increase is more pronounced in the second controls than in the experimental animals. The difference in the weights of the femora of these two groups becomes even more marked when it is considered that the rats receiving irradiated ergosterol showed approximately twice as much growth during the experimental period as did the second controls. There was little difference in the densities of the roentgenograms of the bones of these animals. Taken as a group, the bones of the animals receiving respectively 5850 and 7800 vitamin D units daily were about 2 per cent more dense than the bones of either of the control groups or of the animals which received a daily dose of irradiated ergosterol equivalent to 3900 vitamin D units. Although this difference is outside the experimental error it is too slight to be of any importance.

Table 3, which records the results of the blood analyses, shows that there was little difference in the levels of blood phosphorus of any of the groups, but that the serum calcium of the control animals was markedly below that of either group receiving irradiated ergosterol. Although there was only a very small amount of calcium in the diet, the liberal quantities of vitamin D were able to maintain an approximately normal level of calcium in the blood stream, which appears to have been due to a more economic utilization of the small amount in the diet rather than a withdrawal of

calcium from the bones. No decrease in absolute amount of bone ash was detectable, nor was there any appreciable increase although normal concentrations of blood calcium and phosphorus were maintained.

Sections from the tibiae and femora of these animals were prepared in the same manner as were those of the older animals except the bones were decalcified for only 15 minutes. No sections were prepared from the animals in group I (those killed at the beginning of the experiment).

The bones grossly were quite abnormal for rats of approximately 120 days of age. They were distinctly smaller than normal and very soft. Histologically two very different appearances were found.

The sections from the animals in group II uniformly showed a marked overgrowth of osteoid tissue (fig. 5). The epiphyseal cartilages were prominent, but quite regular in structure with a moderate zone of provisional calcification. The metaphyses showed a marked proliferation of spongy bone, consisting of many spicules of moderately well calcified bone

TABLE 3

Effect of irradiated ergosterol on the levels of blood calcium and phosphorus of young rats on a diet low in calcium

	DAILY DOSE IRRADIATED ERGOSTEROL*	Ca PER 100 cc. SERUM	P PER 100 cc. SERUM
		mgm.	*mgm.*
Control animals	0	6.58	6.30
Experimental animals	3,900	11.18	5.46
Experimental animals	5,850	11.33	6.76

* Dosage expressed in Steenbock units.

overlaid by broad zones of uncalcified osteoid tissue. In the epiphyses there was a slight rim of calcified bone underlying the joint cartilage and a few bony spicules running through the marrow cavity. In both sites the bone was covered by a broad layer of osteoid tissue. The diaphyses showed cortical bone composed of a thin layer of calcified bone overlaid by osteoid tissue. Even with the thick layer of osteoid tissue the cortical bone was thin. It also was quite irregular showing numerous indentations and fenestrations filled with marrow. Throughout all parts of the bones osteoblasts were extremely numerous. The whole picture was one of marked overgrowth of totally uncalcified osteoid tissue, especially in the metaphyses. The appearance was that of rickets, due it is believed to the dietary deficiency of calcium.

In striking contrast to the above picture was that found in the sections from the bones of the animals in group III. In this group the marked proliferation of osteoid tissue was lacking (fig. 6). The epiphyseal carti-

lages showed a more marked zone of provisional calcification and were narrower than those of group II. In the metaphyses bone trabeculae were less numerous, more delicate, and more deeply blue staining. The epiphyses showed a thin rim of deep blue staining bone and delicate septa. The cortical bones of the diaphyses were quite thin, moderately stained with hematoxylin, and irregular, showing indentations and fenestrations filled with marrow. In contrast to group II osteoid tissue was very inconspicuous, although a thin layer overlay the calcified bone practically everywhere. Osteoblasts were found to be fully as numerous as in group II. Osteoclasts which were uncommon in group II were moderately numerous.

The higher relative ash content found in the bones of the rats in group III is evidently correlated with the histological predominance of calcified bone and the paucity of osteoid tissue. The lower ash percentage found in group II similarly is to be accounted for histologically by the abundance of uncalcified osteoid tissue.

It has been frequently suggested that vitamin D, in addition to favorably influencing bone calcification, plays other important rôles in the physiology of the animal body. In this experiment it appears that vitamin D increased growth, tended to prevent the over production of organic matrix of the bone (osteoid tissue) and maintained the normal level of blood calcium without materially increasing calcification. It is difficult to determine whether the epiphyses of the animals receiving irradiated ergosterol were more calcified than those of the second group of controls because the latter showed such an extensive overgrowth of osteoid tissue. Chemical analyses showed no increase in the absolute amount of ash during the experimental period; therefore if the irradiated ergosterol did increase the calcification of the epiphyses it must have done so at the expense of the calcium salts of the other parts of the bone. This action of vitamin D has been suggested by Brown and Shohl (1930).

The increase in osteoid tissue apparently increased the strength of the bones of the animals of the second control group, as these animals had less trouble in locomotion than did the animals which received the irradiated ergosterol. In the latter case the vitamin D preparation appears to have prevented the overgrowth of osteoid but as it was impossible to produce calcification due to the low level of calcium in the diet, the bones of these animals were weaker than those of the controls. Since there was no decrease in absolute amount of ash this difference in apparent strength of the bones presumably was not due to a withdrawal of calcium salts but instead due to the lack of osteoid tissue on the part of the animal receiving irradiated ergosterol.

As stated above, only one animal of this series showed any of the usual symptoms of irradiated ergosterol intoxication. This animal died on the

66th day of the experimental period and its femur had a lower percentage of ash than that of any other animal. The relative amount of ash, however, was less than 1 per cent below that of one of the animals of group II and only slightly more than 2 per cent below that of another. Since the only rat which died of irradiated ergosterol intoxication had the lowest percentage of ash as well as the lowest absolute amount, and was the only animal showing marked toxic symptoms, it was very suggestive that inorganic constituents had been withdrawn from the bone. Consequently the experiment was repeated with a small group of young animals. Six rats of the same litter (5 males and 1 female) were placed on the corn-sodium chloride diet at 28 days of age. Seven days later one of the males was killed, the daily administration of irradiated ergosterol to 3 males and 1 female was begun and the remaining male was continued on the original diet without supplement. The animal which was killed at the beginning of the experiment again showed the highest percentage ash, but the ash content of the femur of the animal which was continued throughout the experimental period without any vitamin D supplement fell between those of the animals receiving irradiated ergosterol. As these results were not very conclusive no further experiments were conducted with young animals. In a second publication by Light, Miller and Frey (1931) data obtained on young rats were presented which indicate a marked withdrawal of ash from the bones. However, these authors do not distinguish between actual withdrawal of inorganic constituents and the failure of these substances to be deposited in the growing bone.

SUMMARY

The femora of rats, ranging in age from 68 to 138 days at the beginning of the experiment and given sufficient irradiated ergosterol to produce death, but only after a period of 6 to 8 weeks, showed very marked degenerative changes. The bones were very porous due to the removal of organic as well as inorganic matrix. Apparently this destruction was the result of osteoclastic activity as shown by histological studies. The bones were very soft and fragile. Chemically they showed a relative decrease in ash content which was apparently due to subsequent growth of osteoid tissue following the absorption of the bone matrix. Because of the removal of organic as well as inorganic material it appears that the action of irradiated ergosterol when given in large doses is not a simple withdrawal of calcium salts from the bone as suggested by various investigators.

Large but non-toxic doses of irradiated ergosterol given to young rats on a diet low in calcium did not materially change the absolute amount of ash in the femora. The relative amounts of ash were greater in the bones of the animals receiving irradiated ergosterol than in the bones of rats continued on the basal diet without supplement, but the difference was due

to a greater development of osteoid tissue in the bones of the latter group. It seems that vitamin D under these conditions has prevented the overgrowth of osteoid tissue without increasing calcification due to the lack of calcium in the diet. The concentration of blood calcium remained about normal in the animals receiving the vitamin D preparation but fell below 7 mgm. per 100 cc. of serum in the animals which were given the basal diet only.

BIBLIOGRAPHY

Brown, H. B. and A. T. Shohl. 1930. Journ. Biol. Chem., lxxxvi, 245.

Jaffe, H. L., A. Bodansky and J. E. Blair. 1931. Arch. Path., xii, 715.

Jones, J. H. and G. M. Robson. 1931. Journ. Biol. Chem., xci, 43.

Kreitmair, H. and U. Hintzelmann. 1928. Arch. Exp. Path. u. Pharm., cxxxvii, 203.

Light, R. F., G. E. Miller and C. N. Frey. 1931. Journ. Biol. Chem., xcii, 47.

Taylor, N. B., C. B. Weld, H. D. Branion and H. D. Kay. 1931a. Can. Med. Assoc. Journ., xxiv, 763.

1931b. Can. Med. Assoc. Journ., xxv, 20.

Watchorn, E. 1930. Biochem. Journ., xxiv, 1560.

Wiskott, A. 1930. Zeitschr. f. Kinderheilk., xlix, 79.

OBSERVATIONS ON THE RE-FORMATION OF CEREBROSPINAL FLUID AND AQUEOUS HUMOR

Experiments on Dogs with Certain Organic Dyes

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Opinions are still variant regarding the mode of production and avenues of circulation of both the cerebrospinal fluid (Weed, 1; Levinson, 2; Walter, 3) and the aqueous humor (Duke-Elder, 4; Yudkin, 5; Friedenwald and Pierce, 6). The common embryonic derivation of the encasing morphologic structures of these fluids and the close similarity of their physicochemical properties, warrant the current assumption that some analogy if not similarity might exist in their function, mode of formation, and circulation (Wegefarth and Weed, 7). The present studies were undertaken to obtain data on the mechanism governing the re-formation of these fluids. The problem was approached by an indirect method. Various organic dyes were given intravenously, then the major portions of both the liquor and the humor were withdrawn and examined. Subsequently, after a given time interval, the re-formed liquor and humor were again tested for the presence of the dye administered. The results obtained by this method suggest that the mechanism governing the re-formation of the cerebrospinal fluid differs from that of the aqueous humor.

Method. Dogs were anesthetized with nembutal (about 0.6 cc. of a 5 per cent solution per kilogram of body weight intraperitoneally) and under aseptic precautions cisternal puncture was performed. Then the selected dye, usually 2 cc. of a 1 per cent solution per kilogram of body weight, was injected into the superficial leg vein. Fifteen minutes later a given amount of cerebrospinal fluid, approximately 0.75 cc. per kilogram of body weight, was slowly withdrawn, and about 0.5 cc. of aqueous humor was removed from the left eye by paracentesis. One hour later samples of the aqueous humor from the right eye, of the re-formed humor from the left eye, and of the cerebrospinal fluid were obtained. The fluids were then examined for the presence of the dye administered.

The following ten dyes were used: phenolsulphonphthalein, uranin, eosin, erythrosin, rose bengal, martius yellow, trypan blue, acid fuchsin, methyl blue and methylene blue. A number of these were selected from

TABLE 1

The appearance in the canine aqueous humor and cerebrospinal fluid of various organic dyes administered intravenously in doses of 20 mgm. per kilogram of body weight

DOG	WEIGHT	APPROXIMATE CONCENTRATION OF DYE IN					
		Aqueous humor				Cerebrospinal fluid	
		Left eye		Right eye		First	Second
		First	Second	First	Second		
Phenolsulphonphthalein							
	kgm.						
D-22	10	None	Trace	None		None	None
D-43	8.6	None	Trace	None		None	None
D-49	5.5	None	Trace	None		None	None
Uranin							
D-54	10	Trace	1:150,000	Trace		Trace	Trace
D-55	9	Trace	1:300,000	Trace		Trace	Trace
D-56	10.4	Trace	1:300,000	Trace		Trace	Trace
Eosin							
D-14	10	None	Trace	None		None	None
D-44	12	None	Trace	None		None	None
D-46	9.3	None	Trace	None		None	None
Erythrosin							
D-12	9.5	None	Trace	None		None	None
D-19	18	None	Trace	None		None	None
D-20	10	None	Trace	None		None	None
Rose bengal							
D-7	12	None	1:15,700	None	Trace	None	None
D-9	9	None	1:37,500	None		None	None
D-10	10.5	None	1:35,000	None		None	None
Martius yellow							
D-29	25	None	Colored	None		None	None
D-30	11.4	None	Colored	None		None	None
D-48	11.5	None	Colored	None		None	None
Trypan blue							
D-34	9.6	None	1:12,600	None		None	None
D-36	13.8	None	Colored	None		None	None
D-50	6.7	None	Colored	None	1:28,000	None	None
Acid fuchsin							
D-51	19.6	None	1:100,000	Colored	Colored	None	None
D-52	9.4	None	1:100,000	Colored		None	None
D-53	12	None	1:60,000	Colored		None	None

TABLE 1—*Concluded*

DOG	WEIGHT	APPROXIMATE CONCENTRATION OF DYE IN					
		Aqueous humor				Cerebrospinal fluid	
		Left eye		Right eye		First	Second
		First	Second	First	Second		
Methyl blue							
	kgm.						
D-39	8	None	1:12,000	None		None	None
D-40	10	None	Colored	None	1:18,000	None	None
D-45	11.3	None	1:9,000	None		None	None
Methylene blue							
D-4	21	None	None	None	None	None	None
D-5	29	None	None	None	None	None	None
D-47	8.6	None	None	None	None	None	None

the dyes used by Wittgenstein and her collaborators who studied extensively the removal from the blood of substances given intravenously (8), the permeability of the meninges (9), the transportation of substances from the blood to the aqueous humor (10) and the influence of the thyroid gland on the permeability of the blood-liquor and blood-humor barriers (11). The phthalein and fluorescein derivatives—phenolsulphonphthalein, uranin, eosin, erythrosin, and rose bengal—have such an intense color that no special chemical method is required for their determination in the liquor or humor. The same may be said of martius yellow, and trypan blue. Acid fuchsin and methyl blue were determined by adding one volume of glacial acetic acid to the fluid, methylene blue by the method used by Halpert and Hanke (12).

EXPERIMENTAL OBSERVATIONS. The data of the experiments are gathered in table 1. The results recapitulated briefly are as follows: 1. *Phenolsulphonphthalein, eosin, erythrosin, rose bengal, martius yellow, trypan blue* and *methyl blue* did not appear in either the humor or the liquor fifteen minutes after intravenous administration. The respective dye was also absent in the humor of the second eye removed one hour later. It was, however, invariably present in the re-formed humor of the first eye but absent in the re-formed cerebrospinal fluid. 2. *Uranin* appeared in traces in both the humor and the liquor fifteen minutes after intravenous administration. One hour later the dye was present in similar quantities in the humor of the second eye. The concentration of the dye in the re-formed humor of the first eye was invariably much greater than in any of the other samples, while the concentration of the dye in the re-formed liquor remained about the same. 3. *Acid fuchsin* did not appear in either

the humor or the liquor fifteen minutes after intravenous administration. The dye was detected in the humor of the intact eye one hour later. Its concentration, however, was distinctly less than that of the re-formed humor of the first eye. The re-formed liquor did not contain the dye. 4. *Methylene blue* did not appear in either the humor or the liquor fifteen minutes after the injection nor in any of the subsequent samples (13).

COMMENT. The observations just presented strongly suggest that there is a difference in the mechanism which governs the re-formation of the cerebrospinal fluid and the aqueous humor. We are, however, not prepared to state the nature of the difference. A critical review of our method and the data obtained by it suggests that the fluid obtained at the second paracentesis was not true aqueous humor and thus not comparable to the second sample of cerebrospinal fluid.

In the experimental procedures meticulous care was exercised to avoid injury of any structure which might cause alteration of the fluids or would interfere with their re-formation. If bleeding was encountered, the experiment was discarded. It was soon learned that about 0.75 cc. per kilogram of body weight was the amount of cerebrospinal fluid which could be removed with impunity by cisternal puncture. The fluid was withdrawn slowly and with several interruptions to allow for readjustment of pressure. Invariably the intracranial pressure was positive at the start since the liquor would flow freely upon removal of the stilette. At each interruption during the removal of the fluid, air would rush in to render the negative pressure atmospheric. We assumed that by this procedure conditions were created about the ventricular surface of the blood-liquor barrier which favored the passage of substances from the blood. In attempting to produce an analogous situation in the eye, only a fraction of the obtainable aqueous humor, not more than 0.5 cc., was removed. This procedure created conditions about the chamber surface of the blood-humor barrier which not only favored theoretically but permitted actually the passage of certain substances from the blood ("plasmoid aqueous" of Duke-Elder)—a fact known to investigators in this field. The fluid replacement in the anterior chamber of the eye was prompt, that in the subarachnoidal space slow.

The differences in the mode of the replacement of the humor and liquor just mentioned are easily observed and well illustrated by the experiments in which dyes regularly appeared in the re-formed humor but not in the liquor. The evidence is reinforced by the data obtained with acid fuchsin. This dye, as may be recalled, eventually appeared spontaneously in the aqueous humor of the intact eye but could not be detected in any of the samples of the cerebrospinal fluid.

SUMMARY AND CONCLUSION

Various organic dyes were given intravenously to dogs, in doses of 20 mgm. per kilogram of body weight. Fifteen minutes later a given amount of cerebrospinal fluid, approximately 0.75 cc. per kilogram of body weight, was slowly withdrawn by cisternal puncture, and about 0.5 cc. of aqueous humor was removed from the left eye by paracentesis. One hour later samples of the aqueous humor from the right eye, of the re-formed humor of the left eye, and of the cerebrospinal fluid were obtained and examined for the presence of the dye administered.

1. Phenolsulphonphthalein, eosin, erythrosin, rose bengal, martius yellow, trypan blue, and methyl blue did not appear in the aqueous humor or the cerebrospinal fluid but the respective dye was invariably present in the re-formed humor of the first eye.

2. Uranin appeared in traces in the aqueous humor and the cerebrospinal fluid. The concentration of the dye in the re-formed humor of the first eye was invariably much greater than in any of the other samples.

3. Acid fuchsin did not appear in the aqueous humor or the cerebrospinal fluid fifteen minutes after its administration. The dye was detected in the humor of the intact second eye one hour later. However, the concentration of the dye was distinctly less than that of the re-formed humor of the first eye. The re-formed liquor did not contain the dye.

4. Methylene blue did not appear in the aqueous humor or cerebrospinal fluid fifteen minutes after the injection nor in any of the subsequent samples.

The above data favor the view that the mechanism governing the re-formation of the aqueous humor differs from that of the cerebrospinal fluid.

BIBLIOGRAPHY

(1) Weed, L. H. Physiol. Rev., 1922, ii, 171.
(2) Levinson, A. Cerebrospinal fluid in health and in disease. St. Louis, C. V. Mosby Company, 1923.
(3) Walter, F. K. Die Blut-Liquorschranke. Leipzig, Georg Thieme, 1929.
(4) Duke-Elder, W. S. , Brit. Journ. Ophthalmol., 1927, Monogr. Suppl. III.
(5) Yudkin, A. M. Arch. Ophthalmol., 1929, i, 615.
(6) Friedenwald, J. S. and H. F. Pierce. Arch. Ophthalmol., 1932, vii, 538.
(7) Wegefarth, P. and L. H. Weed. Journ. Med. Res., 1914, xxxi, 167.
(8) Wittgenstein, A. und H. A. Krebs. Pflügers' Arch., 1926, ccxii, 268.
(9) Wittgenstein, A. und H. A. Krebs. Zeitschr. f. d. ges. exper. Med., 1926, xlix, 553.
(10) Gaedertz, A. und A. Wittgenstein. Graefe's Arch. f. Ophthalmol., 1927, cxix, 397; 1928, cxix, 755.
(11) Gaedertz, A. und A. Wittgenstein. Zeitschr. f. d. ges. exper. Med., 1931, lxxviii, 616.
(12) Halpert, B. and M. T. Hanke. This Journal, 1929, lxxxviii, 351.
(13) Halpert, B., W. J. German and D. R. Mills. Anat. Rec., 1932, lii, 56.

THE INHIBITION OF LACTATION IN RABBITS WITH LARGE AMOUNTS OF OESTRIN

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Since the publication of the findings of Grüter and Stricker (1) in Germany, and of Corner (2) in this country, it has seemed to be established that the anterior lobe of the hypophysis contains a substance capable of producing milk secretion in rabbits, and that this substance may be called the true lactation hormone. Corner in his paper refers to the numerous reports in the literature of the effect of both oestrin and progestin upon the mammary glands, and concludes from their results and his own that, although mammary hypertrophy may result, true lactation similar to that found in postpartum animals cannot be brought about by these hormones. Although the important work of Grüter and Stricker and of Corner indicates that the anterior hypophysis contains the factor directly responsible for lactation, it has seemed to us possible that the ovarian hormones might indirectly be concerned in the mechanism of milk secretion through their action upon the hypophyseal gland. Thus far a number of experiments in this laboratory have suggested some such mechanism, but an almost equal number of failures to produce real lactation have indicated that entirely opposite effects may result merely by varying the dosage or the duration of injections.

In the course of this work we have repeatedly found evidence for the inhibitory effect of very large amounts of oestrin upon lactation in rabbits. This possibility is suggested by the well known fact that in humans the termination of pregnancy, together with the removal of the placenta, a source of tremendous amounts of oestrin, is rapidly followed by the secretion of milk. Hammond (3) observed lactation in rabbits when the pregnancy was removed any time after the 12th day. Fellner (4), Bauer (5) and Brühl (6) have noted that milk secretion is inhibited as long as large amounts of oestrin circulate in the blood. Direct experimental evidence, however, for the inhibition of lactation by the administration of oestrin seems to be lacking.

EXPERIMENTAL OBSERVATIONS. In the case of four rabbits it was noted

that no lactation occurred after the termination of pregnancy. In three of these laparotomy was performed and attached, unabsorbed remnants were found in the uterus, which were shown by microscopic examination to be living placental tissue. The ovaries of all three contained large active corpora lutea.

Rabbit 306 was given an injection of an extract of the urine of pregnancy (500 R.U. Parke, Davis & Company "Antuitrin S") on the 16th day of gestation. On the 19th day miscarriage was indicated by blood from the vagina, but no secretion appeared in the breasts. (In our experience, as in Hammond's (3), lactation always follows miscarriage in rabbits.) On the 31st day, the breasts still being dry, the animal was explored. Its ovaries were full of large active corpora lutea and the uterus contained small attached placental remnants. The uterus was removed and two days later lactation started and lasted for 23 days.

It would appear that the placenta contains some substance which inhibits lactation. Later experiments indicated that this inhibitory factor might be oestrin, although it has yet to be demonstrated that the rabbit placenta contains sufficiently large amounts of this hormone.

An entirely different interpretation of these findings in rabbits with placental remnants and persistent corpora lutea might be argued. Hammond (3) is of the opinion that progestin is the hormone primarily concerned in mammary development and that the removal of this stimulating influence results in secretion. He believes that the lactation which follows the removal of pregnancy in rabbits after the 12th day is due to the regression of the corpora lutea. We have found, however, that lactation ensues immediately after the removal of the pregnancy, while the corpora lutea still appear active. In view of this fact and considering the above observations, it seems more logical to conclude that the placenta itself contains the hormone which inhibits milk secretion as well as the factor concerned in maintaining luteinization of the ovaries (except in pseudopregnancy, in which some other mechanism must function).

Spontaneous lactation often occurs in rabbits even when they are isolated, so that the possibility of ovulation and pseudopregnancy presumably is eliminated. Corner, in a personal communication, stated that this had been his experience. Eight isolated normal does showed spontaneous lactation during the course of one summer in this laboratory, the amount of milk varying from small drops to streams and the duration varying from one to four months. (With one exception we have seen this phenomenon only during the summer months, and it has never occurred in spayed rabbits, which may be evidence for the effect of ovarian hormones upon the pituitary factor.) It was found that this spontaneous lactation was inhibited by injections of oestrin.

One of these rabbits, no. 194, received subcutaneously 16 cc. of theelin (50 R.U. per cc.—Parke, Davis & Company) over five days. This animal was showing milk

in streams when the injections were started. The milk decreased markedly until three days after the last injection. Profuse lactation then recurred and continued for the following four months.

Rabbit 182 began showing drops of milk spontaneously on the 5th of June. On the 26th it was still lactating and was given 50 cc. of theelin over the following eight days. The milk decreased until by July 3rd there was only the slightest possible trace of watery secretion. On the 9th of July real milk reappeared and lasted until the 24th, when 18 cc. of theelin were injected. By the 28th the breasts were entirely dry. Four days later drops of real milk were again being secreted.

It was also found that the administration of large amounts of oestrin would prevent lactation after delivery, death of pregnancy or miscarriage.

Rabbit 219 delivered prematurely on the 20th day of pregnancy and the litter died. The following day streams of milk were found in the breasts. Over the following six days 18 cc. of theelin were injected subcutaneously with a marked decrease in the mammary secretion, until on the 6th day only the slightest possible trace of cloudy material could be expressed. Five days after the last injection, and eleven days after delivery, however, lactation began again and continued for 24 days.

The termination of pregnancy any time after the 20th day in untreated rabbits results in immediate lactation, which lasts at least 20 days, even when there is no suckling.

Two rabbits, palpably pregnant, received theelin on the 20th day, which killed the pregnancy. One of these, no. 174, had a single intravenous injection of 10 cc. It began to lactate two days later and continued for one month. The other rabbit, no. 189, had 10 cc. of theelin on the 20th day and 3 cc. daily for the following six days. No secretion was found until five days after the last injection, when a small amount of watery material was present. There was still less mammary activity five days later, when the animal died. At autopsy the ovaries were found to contain old pale corpora lutea. No products had been retained in the uterus to account for the absence of milk.

Rabbit 182, 20 days pregnant, had six viable fetuses removed by hysterectomy. The animal received 3 cc. of theelin daily for five days beginning the day before operation and showed no mammary secretion until ten days after the last injection of theelin, i.e., fourteen days after the termination of pregnancy. Lactation then set in and lasted for seventeen days.

Rabbits 190 and 191 miscarried about the 20th day of pregnancy following the subcutaneous injection of an extract of sheeps' hypophyses (Parke, Davis & Company). This was the same preparation used by Corner to produce lactation in recently spayed rabbits. Rabbit 190 received two 10-cc. injections on successive days, followed immediately by miscarriage and profuse lactation, which lasted 20 days. Number 191 also had two 10-cc. injections on successive days, followed by miscarriage, but it had in addition 3 cc. of theelin daily for seven days, starting the day of the first injection of hypophyseal extract. It never showed more than a small trace of milk and by the 14th day after miscarriage its breasts were dry and remained so.

With the idea of producing repeated crops of corpora lutea in late pregnancy, three rabbits, nos. 162, 163 and 169, were given five to nine 10-cc. injections of antuitrin S between the 15th and 24th days. In all three laparotomy disclosed that death of the pregnancy had followed. (The ovaries were tremendously luteinized and enlarged,

an effect which has also been observed, although not to such a marked degree, in pseudopregnant rabbits after repeated injections of antuitrin S.) Numbers 162 and 169 received in addition between 1500 and 2000 R.U. of oestrin (theelin and Schering Corporation progynon) in repeated daily injections which were started at the same time as the antuitrin S and continued for two days after the last injection of antuitrin S. During the following month and a half no. 162 never lactated at all and no. 169 showed only the slightest possible trace of secretion, which lasted only a few days. Number 163, on the other hand, having received no oestrin, began lactating profusely after the 5th injection of antuitrin S. Milk was present for the following 30 days.

These observations demonstrate that it is possible to inhibit postpartum lactation with oestrin and to prevent it entirely provided large doses are given or the injections started before secretion has begun.

In the rabbit pseudopregnancy may be produced by any means which causes ovulation and corpus luteum formation without fertilization of the ova. Whether instigated by coitus with a vasectomized buck, intravenous injection of antuitrin S or subcutaneous injection of hypophyseal extract, pseudopregnancy is followed by lactation, which usually appears about 20 days after ovulation and lasts from three weeks to two months. We have been able to inhibit this lactation with theelin.

Rabbit 168, just beginning to show a small amount of milk after a pseudopregnancy, had 6 cc. of theelin over three days. The milk decreased until the breasts were entirely dry. Five days after the last injection milk in small amounts reappeared and was present for two months.

Rabbit 308 started showing milk 24 days after an ovulation. It had been lactating for 12 days and milk could be expressed in streams when 40 cc. of theelin were given over two days. On the third day only a small drop of milk was found and five days later the breasts were dry and remained so.

It is not always possible, however, to stop lactation or even markedly inhibit it with injections of theelin. Our failures have been in rabbits that had already been lactating for some time after delivery. In two such cases as much as 4000 R.U. of oestrin over six days had no convincingly inhibitory effect upon the amount or duration of the secretion.

Another line of evidence for the inhibition of lactation with oestrin was procured in experiments in which large amounts of theelin were given together with the same hypophyseal extract mentioned above. In using this material we found that different lots varied greatly in potency and that the lactation factor was less stable than the gonad-stimulating factor, for an extract that still tested high in its ability to promote luteinization in the ovaries of immature rats was completely ineffective in producing lactation in rabbits. This instability makes it difficult to evaluate the results of experiments performed two or more months apart. The following protocols, however, seem to indicate that the lactation brought on by injections of this preparation can be prevented by the simultaneous administration of large amounts of theelin.

Rabbit 179, a normal doe, received 80 cc. of hypophyseal extract and 24 cc. of theelin over five days. During the following 15 days there was never more than the slightest possible trace of milk. Two months later 60 cc. of this same extract produced immediate profuse lactation in two different does, one normal and the other recently spayed.

Number 257, spayed, and no. 293, normal, were each given 40 cc. of hypophyseal extract over four days. These two experiments were performed simultaneously and with the same extract. Number 293 also received 40 cc. of theelin during the same period. Number 257 began lactating on the fourth day of the experiment and by the seventh day had milk in streams, which continued in decreasing amounts for 13 days more. In following the mammary activity of no. 293 over the same period, on the other hand, only drops of watery material could be expressed.

It had previously been demonstrated that lactation immediately followed the administration of hypophyseal extracts in normal rabbits, indicating that the presence of the ovaries does not prevent lactation produced in this manner.

Discussion. Discussion of the mechanism of this inhibitory action of large amounts of oestrin upon lactation can at present be only theoretical. Fluhmann (7), in his excellent review of the literature on the interrelationship of the anterior hypophysis and the ovaries, refers to numerous investigators who have demonstrated a definite effect of oestrin upon the hypophyseal gland in respect to the latter's ability to stimulate the gonads, to promote growth and to produce uterine bleeding. Changes in the histology of the anterior pituitary after the administration of oestrin have also been reported. Since it has been established that the hypophysis contains the true lactation hormone, the possibility suggests itself that oestrin may influence mammary secretion through its action upon the pituitary. In dealing with these hormones it is more and more being realized that dosage is an important consideration. For example, small amounts of oestrin are necessary in order to obtain progestational proliferation of the endometrium of the rabbit with progestin (8), whereas large amounts of oestrin completely inhibit the process (9). It is entirely possible that small quantities of oestrin might affect the anterior hypophysis in such a way as to increase its ability to promote mammary secretion and that large doses might cause changes resulting in inhibition of the lactation factor. On the other hand, since physiological amounts of oestrin are involved in mammary growth preparatory for secretion, it may be that excessive doses exert their inhibitory action directly upon the breasts.

SUMMARY

Observations of rabbits with retained living placental remnants have indicated that this tissue contains a substance capable of inhibiting the secretion of milk.

The administration of large amounts of oestrin to postpartum rabbits,

to those exhibiting spontaneous lactation and to those showing milk following pseudopregnancy, has resulted in a marked decrease or even total disappearance of the secretion. The administration has been found more effective in preventing the appearance of milk under the same circumstances. The lactation which immediately follows injections of hypophyseal extract has also been inhibited by large quantities of oestrin.

The possibility of an interrelationship between oestrin and the lactation factor of the pituitary is discussed.

BIBLIOGRAPHY

(1) GRÜTER, F. AND P. STRICKER. 1929. Klin. Wochenschr., viii, 2313.
(2) CORNER, G. W. 1930. This Journal, xcv, 43.
(3) HAMMOND, J. 1917. Proc. Roy. Soc. B., lxxxix, 534.
(4) FELLNER, O. O. 1931. Klin. Wochenschr., x, 1164.
(5) BAUER, A. W. 1931. Med. Klin., xxvii, 1390.
(6) BRÜHL, R. 1930. Zentralbl. Gynökol., liv, 192.
(7) FLUHMANN, C. F. 1931. Amer. Journ. Obst. and Gynec., xxii, 803.
(8) HISAW, F. L. AND S. L. LEONARD. 1930. This Journal, xcii, 574.
(9) ALLEN, W. M. 1932. This Journal, c, 650.

CONDITIONED INHIBITION OF WATER DIURESIS

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Reports of conditioned water diuresis reflexes have been made by Bykow and Berkmann (1927, 1930, 1931), Grossmann (1929), and Marx (1931a, 1931b). The successful experiments reported by Bykow and Berkmann, the less conclusive work of Grossmann, and the negative results of Marx indicate in some measure the complexities involved in the production of such a conditioned reflex.

Indeed, the latter investigator reported (1931b) the formation of a conditioned diuresis reflex in only one of the four dogs used, and, even in the successful case, "the expectation was not fulfilled during the first five months." Contrast this with the results of Grossmann (1929) who reported the presence of the reflex "after daily experiments for one week," and the description by Bykow and Berkmann of successful conditioned diuresis reflexes "after fifteen to twenty trials" (1927), "after sixteen rectal injections of water" (1930), and "after several injections of water" (1931). Marx (1931a), employing the method of Bykow and Berkmann on six dogs, was unable to produce a conditioned diuresis reflex even after twenty rectal injections of water. The latter investigators also reported (1931) that conditioned diuresis reflexes are not affected by denervation of the kidneys, and concluded that the reflex path of conditioned diuresis consists of closely related nervous and humoral components. It was during the course of experiments attempting to confirm the observations of Bykow and Berkmann that the following case of conditioned inhibition of water diuresis developed. It is described because it may furnish an explanation of the difficulty of obtaining positive results in this field.

Experiments were carried out on "Polly", a female police dog (14 kgm.), and "Collie", a female collie (8 kgm.). Permanent bilateral fistulae of the ureters were made on both dogs by Dr. W. M. Firor of the Department of Surgery, to whom grateful acknowledgement is made. In the following discussion we shall limit ourselves to the results obtained with "Polly", the dog which developed conditioned inhibition to the unconditioned stimulus.

On February 24, 1932, bilateral ureteral fistulae were made on "Polly". The dog recovered rapidly from the operation and on March 2 experiments were begun. The dog was given a standard diet of meat and specially

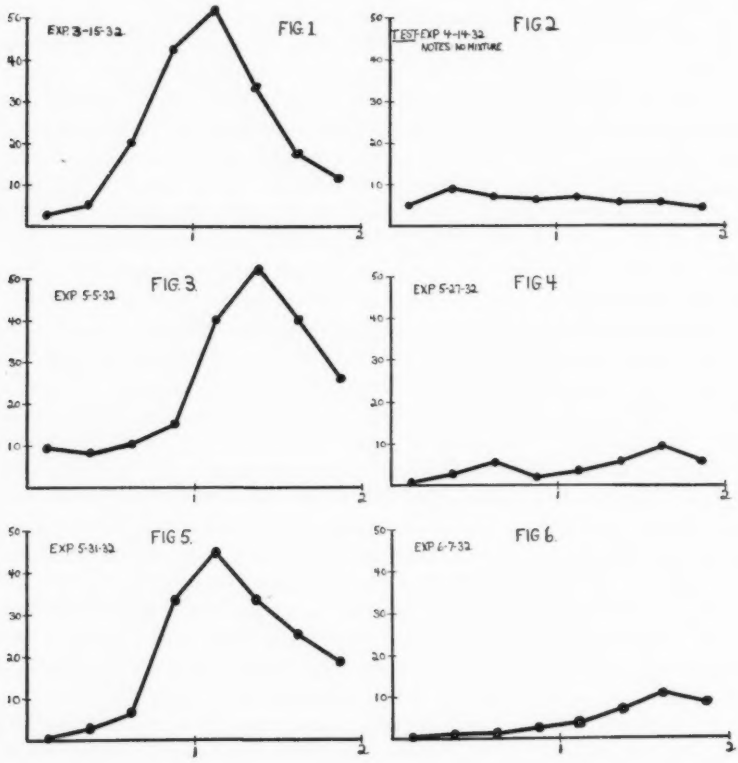
made "bread" containing beef, gravy, cornmeal, and salt, and was fed at approximately 5 p.m. daily, twenty hours before each experiment. The temperature of the experimental room was maintained fairly constant. The water intake was controlled as follows: Water was allowed *ad libitum* from the conclusion of an experiment until 8:30 a.m. of the following day, at which time the water was removed. Experiments were carried out daily (except Sunday) at approximately the same time (1 p.m.) so that the animal had been deprived of water for at least four and a half hours, and possibly longer, depending upon when the last drink was taken. In attempting, in later experiments, to analyze the stimulus complex of inhibition, the dog was restricted to one drink of water (usually 500 cc.) given at the time of feeding. This altered means of water intake lasted two weeks, was followed by a return to the former method, and showed no change in the previous results. The urine drained readily through a funnel strapped to the abdominal wall, and was collected directly into an attached glass reservoir from which it was measured in graduated cylinders at fifteen minute intervals.

At approximately the same time each day the dog was led to the elevator by the same experimenter, carried downstairs, led to the experimental room, placed on a table equipped with a Pavlov stand, and carried through the subsequent experimental procedure. The experimenter was obscured from the dog by a wall made of composition-board, so that the animal could not see the pan until the time of presentation. The funnel was adjusted and the measurement of urine begun immediately after the dog was placed on the table. Fifteen seconds later a buzzer was sounded for fifteen seconds, a pan containing 100 cc. of a 1:4 mixture of milk and water was then presented, and the buzzer was continued for fifteen seconds more. At five minute intervals 100 cc. portions of the mixture were thus presented till, in all, the dog had ingested 500 cc. of the fluid. Experiments, as a rule, lasted two hours, although in some cases they were continued for longer periods. A typical experiment is illustrated in figure 1.

The method outlined above was carried out daily. After twelve days, the first test experiment was made. In this experiment the usual procedure was maintained except that no water-milk mixture was given. Regular experiments were continued daily, being interrupted at intervals by test experiments. On the day that a test experiment was conducted, a regular experiment followed within several hours, so as to reinforce the conditioned stimulus.

After thirty-five experiments a slight conditioned diuresis seemed to have been established (test experiment of 4-14-32, fig. 2). As time went on, the dog appeared to have become conditioned not only to the buzzer (which was to have been the conditioned stimulus) but to the entire experimental procedure, i.e., the presence of the experimenter, the elevator,

entrance into the experimental room, placing on the table, adjustment of the urine-collecting apparatus, etc. Analysis of this complex sequence of stimuli showed that a slight diuresis was brought about by the presence of the experimenter, and by the bringing of the dog to the experimental room. This diuresis (without fluid), however, was not yet entirely satisfactory to



Figs. 1–6. The abscissas represent time in hours and the ordinates rates of urine flow (cc. per 15 min.). The administration of the water-milk mixture was begun at zero time. Figure 1 shows a typical normal diuresis; figure 2, slight conditioned diuresis; figure 3, increase in length of latent period; figure 4, inhibition of diuresis; figure 5, diuresis with changed environment; figure 6, inhibition of diuresis.

prove the possibility of a conditioned diuresis reflex. Regular experiments were continued. Some time later, conditioned inhibition developed.

The experiment of 4–12–32 gave the first indication of inhibition. Although, as usual, 500 cc. of the water-milk mixture had been ingested, the diuresis failed to appear. Occurring after thirty-two experiments had been

carried out, in each of which the ingested 500 cc. had caused a diuresis, this inhibition could not be explained at the time. Three experiments followed in which the usual diuresis was obtained, the next experiment showed inhibition, three more experiments indicated diuresis, and then inhibition again occurred. Inhibition appeared at various intervals thereafter, and the magnitude of the diuresis was strikingly decreased in several other instances. The phenomenon persisted thus until, starting with the experiment of 5–17–32 (after seven previous experiments in which diuresis was exhibited), there followed seven consecutive experiments in which diuresis failed completely. In the attempt to remove this inhibition of the unconditioned response, the next experiment (5–30–32) was carried out as usual, except that the sounding of the buzzer was omitted. This gave little or no change, the inhibition of water diuresis occurring as before. When, however, the experimental procedure was changed, a diuresis was obtained. Upon bringing the dog to a strange room, omitting the use of the table, Pavlov stand, and the buzzer, and upon presenting the 500 cc. of mixture at one time in a strange pan, a marked diuresis occurred (fig. 5). When the 500 cc. of mixture were presented at one time in the regular experimental room (6–1–32), diuresis was also obtained, though not comparable in magnitude to that of the previous experiment. A considerable diuresis also appeared when the mixture was given in the strange room in the usual five 100 cc. portions. Diuresis was brought about, too, after reverting to the use of the experimental room and the usual routine. After another experiment under the same conditions, which resulted in a diuresis of lesser extent, inhibition returned (fig. 6). Thus, inhibition of water diuresis in the regular experimental room was removed by complete change in the procedure, only to have it return again within two days. Reverting to the use of the strange room caused a return of normal diuresis. Continuation of experiments in the strange room effected a decrease in the magnitude of the diuresis even in that room until, eventually, even signs of inhibition began to appear.

Inhibition manifested itself, too, by the effect upon the time of the peak of the curve. Whereas the peak occurred either 60 or 75 minutes after the ingestion of the first 100 cc. of fluid in practically all of the first thirty-five experiments, considerable change was noted after the onset of inhibition. The peak was now delayed to the 90,105, and even the 120 minute point (fig. 3). Removal of inhibition by alteration of the experimental process resulted in the immediate return of the peak to the normal 60 or 75 minute point, where it remained so long as there was no inhibition (fig. 5). With the restoration of inhibition, however, the peak was again delayed, in these cases (expts. of 6–6–32 and 6–7–32) to the 105 minute point (fig. 6). The return to the strange room, etc., removed the inhibition to water diuresis, and the peak was no longer delayed. It follows, then, that the

time of the peak as well as the magnitude of the diuresis may be governed by psychic factors, inhibition delaying the former and decreasing the latter.

SUMMARY

In the course of experiments attempting to produce a conditioned water diuresis reflex, there developed a conditioned inhibition of the unconditioned response. This was characterized by an increase in the latent period of response to water ingestion, a decrease in the magnitude of the response, and finally a total lack of diuresis to water ingestion. A change of the environment of the animal and of the experimental procedure caused the diuresis to reappear.

I desire to thank Dr. E. K. Marshall, Jr., for suggesting the problem, and for advice and aid in its execution.

BIBLIOGRAPHY

Bykow and Berkmann. 1927. Compt. rend. Acad. Sci., Paris, clxxxv, 1214.
1930. Pflüger's Arch., ccxxiv, 710.
1931. Ibid., ccxxvii, 301.
Grossmann. 1929. Klin. Wochenschr., viii, 1500.
Marx. 1931a. Ibid., x, 64.
1931b. This Journal, xcvi, 356.

A STUDY OF THE PHOSPHATES IN THE BLOOD AFTER STRENUOUS MUSCULAR EXERCISE[1]

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Before the realization of the rôle of phosphorus in muscular contraction, numerous experiments were made on the changes of phosphoric acid in blood and urine following exercise, the literature of which has been reviewed by Harvard and Reay (1926). These former studies were made during and after long periods of moderate exercise. Furusawa, Hill, Long and Lupton (1924) and Sargent (1926) have shown that the greatest oxygen debt is obtained by running at maximal speed for a very short period of time. Under these conditions one may assume the largest accumulation of lactic acid in the muscles and the blood. The same should hold for the breakdown of phosphocreatine, that the highest level of inorganic phosphate should be reached in the muscles and in the blood following a short burst of rapid running, for the muscles are working under the maximal anaerobic state that can be obtained in man. With this in view, we have determined blood phosphates in man following a run of sixty yards in ten seconds on a tread-mill[3] and in the blood of dogs following direct and indirect stimulation of their muscles.[4]

METHODS. The method of Fiske and Subbarow (1925) was used for the inorganic and the acid soluble organic phosphates in whole blood.

In order to obtain an indication of the constancy of the phosphates in the blood over a period of one hour with a subject in basal condition, four ten cubic centimeter samples were drawn from an arm vein at fifteen minute intervals with the subject lying quietly in bed. These samples were analyzed for inorganic and organic phosphates. The results for one subject are given in table 1. A similar experiment was made on another subject, the results of which showed similar variations between samples.

[1] A preliminary report of this work was made before the XIVth International Physiological Congress, 1932, Rome, Italy.

[2] Rockefeller Foundation Fellow.

[3] The authors wish to thank the Helen Hartley Jenkins Fund for Medical Research for their financial aid in securing the treadmill.

[4] In some experiments analyses were made of creatine and creatinine in the blood. Realizing the difficulty of interpretation of the results obtained by the picric acid method, these determinations are disregarded.

On the day of an exercise experiment the subject came to the laboratory without breakfast, dressed in a track suit and rested for one hour on a bed beside the tread-mill, at the end of which time one or two 10 cc. samples of blood were drawn from an arm vein for the basal value. Then the subject ran about sixty yards on the tread-mill in ten seconds, returned to the bed as soon as the mill stopped, whereupon four or five samples of blood were drawn during the next hour.

Following the experiments on man, two experiments were made on dogs. The dogs were anesthetized with cyclohexenyl ethyl barbituric acid (Phanodorn) using 80 mgm. per kilo injected intravenously in divided doses, 60 mgm. per kilo followed in twenty minutes by a second injection of 20 mgm. per kilo. In the first experiment a small incision was made through the skin over the gastrocnemius muscle, a bipolar electrode was placed on the exposed muscle and the muscle was stimulated with a tetanic current for a ten second period. Before the stimulation the femoral

TABLE 1

Basal experiment on B. R. March 15, 1932

TIME	PHOSPHATE	
	Inorganic	Organic acid-soluble
	mgm. P per 100 cc. blood	*mgm. P per 100 cc. blood*
10:00	2.94	21.86
10:15	2.88	23.22
10:30	2.88	23.22
10:45	2.78	23.42

vein was exposed and two samples of blood were taken for the resting level. After stimulation, four samples of blood were drawn covering a period of thirty-two minutes. In the second experiment one electrode was fastened to the dog's tail, the other electrode, a large square plate of zinc, was inserted through a skin incision over the upper lumbar vertebrae, the blood samples likewise taken from the femoral vein. This procedure is similar to the one used by Eggleton and Evans (1930) in their study of lactic acid formation following stimulation of both hind legs of a dog. Six ten second periods of tetanic stimulation were used with five second rest periods.

Results. The results before and after exercise are given in tables 2 and 3. In man no significant rise in inorganic phosphate was obtained in any experiment. In each experiment, however, the inorganic phosphate did show a fall, the decrease appearing generally in the second sample taken from seven to thirteen minutes after the run. This fall continued during the hour following the period of exercise. The acid soluble organic phosphate showed a slight rise in each case. In B.R. the acid soluble organic

TABLE 2

SUBJECT: B. R., MARCH 9			SUBJECT: B. R., MARCH 24			SUBJECT: R. S., APRIL 26		
Time	Phosphates		Time	Phosphates		Time	Phosphates	
	Inorganic	Acid-soluble		Inorganic	Acid-soluble		Inorganic	Acid-soluble
minutes			*minutes*			*minutes*		
Basal	3.22		Basal	3.76		Basal	2.46	26.36
2	3.36	23.04	2	3.76		Basal	2.38	25.22
7	3.02	22.48	8	3.52		2	2.50	27.70
22	2.58	21.22	21	3.04		10	2.36	27.14
44	2.30	17.70	40	2.76		26	2.10	26.20
60	2.28	18.32	63	2.54		50	1.98	26.20
SUBJECT: C. G., APRIL 5			SUBJECT: C. G., APRIL 8			SUBJECT: C. G., APRIL 14		
Basal	3.30	26.38	Basal	3.60	26.28	Basal	3.30	25.54
3	3.42	27.98	Basal	3.54	26.34	Basal	3.10	27.24
11	3.38	28.08	3	3.40	28.42	3	3.08	28.12
25	2.94	26.06	13	3.28	27.48	12	3.08	29.38
59	2.70	25.70	29	3.02	26.98	25	2.76	
			59	2.88	26.32	54	2.64	29.36

The time is given from the beginning of the run to the end of the withdrawal of the sample.

Speed of running: B. R. 52 yards in ten seconds, C. G. 63 yards in ten seconds, R. S. 62 yards in ten seconds.

Values for the phosphates are given in milligrams P per 100 cc. of whole blood.

TABLE 3

Experiments on dogs

EXPERIMENT 1			EXPERIMENT 2		
Time	Phosphates		Time	Phosphates	
	Inorganic	Organic acid-soluble		Inorganic	Organic acid-soluble
minutes	*mgm. P per 100 cc. blood*	*mgm. P per 100 cc. blood*	*minutes*	*mgm. P per 100 cc. blood*	*mgm. P per 100 cc. blood*
Resting	3.10	29.50	Resting	4.07	29.95
Resting	3.10	29.00	Resting	4.07	30.83
2	3.10	28.55	2	4.88	33.22
10	3.10	29.20	7	4.40	31.50
20	3.17	29.43	17	4.34	29.96
32	3.14	30.66	30	4.26	31.04

Experiment 1. May 3, 1932. Stimulation of gastrocnemius for 10 seconds.
Experiment 2. May 5, 1932. Stimulation of hind legs for six 10 second periods.

phosphate showed a late decrease, this fall being roughly proportional to the decrease in inorganic phosphate.

In the experiment on the dog with a ten second stimulation, no change was observed in the inorganic or acid soluble organic phosphate. In the second experiment, with six ten second periods of stimulation, a slight but definite rise in the inorganic and organic phosphates was found.

Discussion of results. From our knowledge of the free passage of phosphate in and out of isolated muscles and of the breakdown of phosphocreatine on stimulation of muscles one would expect an increase of phosphate in the blood of man and animals after strenuous muscular exercise.

Several observers have shown that an isolated muscle is permeable to inorganic phosphate. Embden and Adler (1922) found that more phosphate diffused from a fatigued muscle than a resting one. They attributed this to a change of permeability of the muscle fiber during activity. Stella (1928) determined that the inorganic phosphate of a resting muscle is in equilibrium with 8 mgm. P per cent and in the fatigued muscle with 18 mgm. P per cent. As the phosphate content of a fatigued muscle is considerably higher than 18 mgm. per cent Stella concluded that part of the phosphate was incapable of diffusion. Stella observed that the diffusion of phosphate through free solution was more rapid than through muscle. Semeonoff (1931) found a concentration of 50 mgm. P per cent in the Ringer solution was necessary for equilibrium with a fatigued muscle. She concluded that all the phosphate in the fatigued muscle is freely diffusible.

In man, Havard and Reay (1926) found a slight increase in inorganic phosphate in blood following exercise. They attributed this to changes in the lactacidogen content of the muscles, the freed phosphate diffusing out of the muscles into the blood. These experiments are open to several objections. The exercise consisted of running up and down stairs four or five minutes. This type of exercise does not give a maximal oxygen debt. The analyses of inorganic phosphates were made by the Briggs method, a method that has been considerably improved by Fiske and Subbarow (1925). Finally, all the subjects were not in a basal condition at the beginning of exercise. The small increase observed in the inorganic phosphate averaged 10 per cent which may be due in part or wholly to the increased concentration of the blood during exercise. Dill, Talbott and Edwards (1930) found a slight increase in inorganic phosphate in the blood after a run of twenty minutes on a tread-mill, at a rate of 9.3 km. per hour. After taking into consideration the passage of fluid from the blood to the tissues, they concluded that there was an actual transfer of phosphates from the muscles to the blood. The slight rise in organic phosphate observed in our experiments on man may be due to the increased concentration of the blood.

Under the conditions of these experiments on man and dogs one would

expect phosphocreatine to be broken down. A ten second run at maximal speed gives in C.G. an oxygen debt of five to seven liters (Gemmill, 1931) which indicates that the muscles are working under partly anerobic conditions. Nachmansohn (1928) has shown that fifty per cent of the phosphocreatine is broken down by a five second tetanus of isolated frog's muscle in nitrogen. Fiske and Subbarow (1929) obtained 54 mgm. per cent inorganic phosphorus in a cat's gastrocnemius with the circulation intact, an increase of 34 mgm. per cent from the resting level. Stimulation for three minute periods gave practically the same result. However, the resynthesis of phosphocreatine also is very rapid. Nachmansohn (1928) found that thirty per cent of the phosphocreatine was rebuilt in twenty seconds under anerobic conditions and Gemmill (1932) found that the recovery was even more complete when sartorii were stimulated in Ringer's solution.

With the rapid resynthesis of phosphocreatine, the assumption may be made that the inorganic phosphate does not accumulate in the working muscles of man running sixty to seventy yards in ten seconds to such an extent as to give a corresponding increase in blood inorganic phosphorus. Lactic acid accumulates in the muscles under these conditions as the energy for the reconversion of the phosphocreatine comes directly or indirectly from the breakdown of glycogen into lactic acid. Thus lactic acid diffuses into the blood and gives the increase in blood lactate.

Other possible explanations may be suggested. Although permeable in an isolated muscle, the muscles in an intact animal may have different properties and be impermeable to inorganic phosphate. Also, phosphocreatine may not be broken down in muscles under normal conditions. The fact that a slight increase in inorganic phosphate was found in the experiment on the dog where the muscles were stimulated over a long period of time and in the experiments of Dill, Talbott and Edwards (1930) in man exercising for twenty minutes suggests strongly that phosphocreatine is broken down and can diffuse out into the blood if the exercise is continued over a sufficiently long period of time.

Another factor is the excretion of phosphate by the kidney. In all of the experiments on man,a fall of the inorganic phosphate was observed following the exercise. Harvard and Reay (1926) have discussed this decrease of blood phosphate. They concluded that it was due in part to the excretion of phosphate by the kidney and in part to the use of phosphate in the reconversion of lactic acid. So there is the possibility that even under the conditions of fast running for short periods of time there may be a slight amount of phosphate diffusing into the blood, but it would be masked by the greater decrease.

Fiske and Subbarow (1929) found that stimulation of one group of leg muscles did not give an increase of phosphocreatine of the resting group of leg muscles on the opposite side. The reason for this is clear from our

studies, the phosphoric acid in the blood does not increase following exercise to such an extent that there would be sufficient material for formation of phosphocreatine in the resting muscle. This result is in contrast to the reformation of glycogen in resting muscle and liver from the lactic acid carried by the blood from the active muscle.

Conclusions. The chemical changes taking place during and following a run at maximal speed for ten seconds are probably of this nature: Phosphocreatine is broken down into phosphoric acid and creatine but the end products are resynthesized to phosphocreatine very quickly, so quickly that they do not accumulate in sufficient quantity to diffuse into the blood. Glycogen breaks down into lactic acid to supply directly or indirectly the energy for the reconversion of phosphoric acid and creatine into phosphocreatine. As the muscles are not supplied with a sufficient amount of oxygen, lactic acid accumulates in the muscles and diffuses into the blood. The oxidation of the lactic acid after the exercise gives the oxygen debt. In order to maintain the acid base equilibrium, phosphate is excreted by the kidneys and is also used in the reconversion process of lactic acid to glycogen. The last two factors account for the continued fall of the phosphate in the blood that takes place during the hour recovery period. Thus a complete recovery period in man after a ten second run is more than a return of the gaseous metabolism to the basal values, and all the systems that are changed must be considered before the complete story of the physiological effect of short violent exercise in man can be told.

SUMMARY

1. A study was made of the inorganic phosphates and acid soluble phosphates in the blood of man following a fast run of ten seconds on a treadmill and in the blood of dogs following direct and indirect stimulation of their muscles. In man no increase was found in the inorganic phosphates. A slight increase in acid soluble organic phosphates was observed. A late fall of inorganic phosphates continuing throughout the hour recovery period was also found. In the dogs no change was noted in inorganic or in acid soluble phosphates following a ten second stimulation. For six such periods with five second intervals an increase in inorganic and acid soluble organic phosphate was obtained.

From which it is inferred that the breakdown and reconversion of phosphocreatine in man and dogs during violent exercise of short duration is so completely reversible that inorganic phosphate does not accumulate in the muscles sufficiently to give an increase in the blood. Only in cases of prolonged exercise does this increase in the blood occur and then it is of a small magnitude. This is in contrast to the changes that take place in the lactic acid content of the blood following exercise.

BIBLIOGRAPHY

DILL, D. B., J. H. TALBOTT AND H. T. EDWARDS. 1930. Journ. Physiol., lxix, 267.
EGGLETON, M. G. AND C. L. EVANS. 1930. Journ. Physiol., lxx, 269.
EMBDEN, G. AND E. ADLER. 1922. Zeitschr. physiol. Chem., cxviii, 1.
FISKE, C. H. AND Y. SUBBAROW. 1925. Journ. Biol. Chem., lxvi, 375.
1929. Journ. Biol. Chem., lxxxi, 629.
FURUSAWA, K., A. V. HILL, C. N. H. LONG AND H. LUPTON. 1924. Proc. Roy. Soc. B. xcvii, 167.
GEMMILL, C. L. 1931. This Journal, xcviii, 135.
1932. Biochem. Zeitschr., ccxlvi, 319.
HAVARD, R. E. AND G. A. REAY. 1926. Journ. Physiol., lxi, 35.
NACHMANSOHN, D. 1928. Biochem. Zeitschr., cxcvi, 73.
SARGENT, R. M. 1926. Proc. Roy. Soc. B., c, 440.
SEMEONOFF, E. 1931. Quart. Journ. Exper. Physiol., xxi, 187.
STELLA, G. 1928. Journ. Physiol., lxvi, 19.

STUDIES ON THE PHYSIOLOGY OF LACTATION

II. Lactation in the Male Guinea Pig and its Bearing on the Corpus Luteum Problem[1]

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For many years workers interested in the control of mammary gland development and milk secretion have believed that these phenomena were entirely under the influence of the ovarian secretions. The generally accepted theory held that the growth of the glands through puberty and up to the occurrence of a pregnancy was due to the action of oestrin. This development was supposed to be further augmented through the agency of the hormone produced by the corpus luteum of gestation which, according to the classical theory, induced the profound mammary development observed during the final stages of pregnancy. Lactation was supposed to occur following the removal of the lutein influence.

Without considering at this time the inadequacy of this theory to answer the criticisms that may be raised against it, attention is directed to the recent experimental work that has not only presented negative evidence in regard to the rôle supposed to be played by the corpus luteum, but also has shown that the anterior hypophysis is directly concerned. Corner (1930), using virgin rabbits, and Nelson and Pfiffner (1930, 1931), employing immature and mature guinea pigs of both sexes, male and female rabbits, and female rats, have been able to show that prolonged injection of corpus luteum extracts, known to be potent for the production of progestational proliferation, does not induce complete mammary growth and secretory activity. Furthermore, these workers as well as Stricker and Grüter (1929) have presented evidence that secretory activity in the mammary glands may be induced, regardless of the presence or absence of the ovaries, by the administration of anterior pituitary extracts, providing the glands have attained the condition characteristic of the adult non-pregnant female. However, these various workers have not agreed entirely concerning the exact rôle played by the hormone of the corpus luteum in the

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preparation of the mammary glands for the induction of lactation. The present experiments were designed to continue the investigation of the necessity for a lutein stimulus on the mammary glands in preparation for the lactation-inducing stimulus of the anterior pituitary.

The male guinea pig was selected as the principal experimental animal in this study since previous work had shown that while an extremely rudimentary type of mammary development is present in this animal, the glands are quite responsive to developmental stimuli. All males were either castrated or made experimentally cryptorchid, the latter group enabling us to study the possible effect of the male hormone on the growth and function of the mammary glands. Except where otherwise indicated each animal received an ovary implanted in either the kidney or the testicle. In cases of successful incorporation, ovaries furnish a more truly physiological stimulus than is obtained by the parenteral administration of the ovarian hormones. Furthermore, in our experience, ovarian grafts in the male animal normally do not contain corpora lutea although normal follicles are present in functional grafts. The reason for this curious fact is unknown at the present time, but the condition lent itself to our requirements in the present study. By the use of male animals bearing ovaries which produce oestrin in some quantity, but which, in so far as we can determine, do not elaborate the lutein hormone, we were enabled to study the effectiveness of oestrin supplied in a physiological manner, in developing the mammary gland to a condition wherein it will respond to the lactation-inducing stimulus of the anterior pituitary.

It is possible to gauge the functional incorporation and activity of ovarian grafts by examination of the nipples. In a normal male guinea pig the nipples are very small (2 to 3 mm. in height), but they respond readily to oestrin and this fact renders nipple height an index for judging the condition of the ovarian graft. Grafts may function for a time and then undergo degeneration. This occurrence may be recognized by the cessation of nipple growth and usually by a regression in height.

A number of males were injected with an olive oil preparation of oestrin prior to treatment with the hypophyseal extract. The oestrin was prepared by Dr. R. G. Gustavson of the University of Denver to whom the authors express their appreciation. The principal pituitary extract used in this study was prepared in the Research Laboratories of Parke, Davis & Co. and has previously been shown to be active in the lactation-inducing principle. We are deeply indebted to Dr. O. Kamm and Dr. E. P. Bugbee for their sincere coöperation in furnishing us with a liberal supply of pituitary extracts. We are grateful also to Dr. H. B. Van Dyke and Mrs. Z. Wallin-Lawrence for the opportunity to test their pituitary hebin in these experiments.

The general procedure followed in treating animals with the pituitary

extract was to inject subcutaneously 0.5 to 1 cc. twice daily, the period of treatment varying with the individual experiment. In some instances only a single injection was given on each of the last few days before sacrifice. All ovaries and mammary glands were recovered at the close of an experiment and were prepared for histological study.

In no instance could milk or any fluid whatsoever be expressed from the nipples of the guinea pigs used in this study at the time of initiating pituitary treatment. This was true despite the fact that in most of the animals the nipples and glands had attained, through the activity of the ovarian grafts, a size considerably beyond that seen in adult virgin females. Following the initial injection of the pituitary extract all animals were exam-

TABLE 1

Guinea pigs permitted to retain their grafts during the period of pituitary treatment

NUMBER AND TYPE OF ANIMAL	NIPPLE HEIGHT	AGE OF GRAFT	AMOUNT AND PERIOD OF INJECTION	FIRST APPEARANCE OF MILK FOLLOWING THE INITIAL INJECTION	DEGREE OF LACTATION
	mm.	*months*			
Ca.*222	15	3	0.5 cc. 17 times in 10 days	48 hours	Excellent
Ca. 524	4	4	1.0 cc. 10 times in 6 days	72 hours	Good
Ca. 526	11	4	1.0 cc. 9 times in 6 days	48 hours	Excellent
Cr. 449	8	11	0.5 cc. 17 times in 10 days	48 hours	Excellent
Cr. 665	9	3	0.5 cc. 6 times in 4 days	56 hours	Good
Cr. 407	11	11	1.0 cc. 3 times in 36 hours	Colostrum in 24 hours	
Cr. 468	8	10	1.0 cc. 5 times in 54 hours	48 hours	Good
Cr. 567**	7	7	0.7 cc. 10 times in 5 days	70 hours	Good
Fe. 559‡	7	7	1.0 cc. 12 times in 7 days	54 hours	Good

* Ca. = Castrate, Cr. = Cryptorchid.

** Injected with pituitary hebin.

‡ Spayed female with ovarian graft.

ined at 6 to 8 hour intervals. In most instances milk could be expressed within 36 to 70 hours following the initiation of treatment. Milk secretion was usually preceded by a short period during which colostrum was expressible. The various details concerning individual animals are most adequately expressed in table form. Table 1 deals only with animals which were permitted to retain their ovarian grafts throughout the injection period.

Animals 497 and 468 are worthy of mention and further explanation. We realized that the anterior lobe extracts would exert a luteinizing influence upon the ovarian grafts, but we were uncertain with regard to the rapidity of this reaction and its time relation to the occurrence of lactation. The necessity for a closer examination of these unknown factors became

apparent when the treated animals showed the lactation response within a few hours following the initial injection. Accordingly, guinea pig 407 was given only three injections and was killed on the 36th hour following the initial treatment. At the time of sacrifice he had been secreting colostrum for 12 hours and from a study of the glands we are confident that milk would have been produced within 24 hours. Guinea pig 468 was injected five times and sacrificed 54 hours after the start of the experiment. He had been lactating for at least 6 hours. Examination of the ovarian grafts from these animals showed that extensive luteinization had occurred in most of the follicles. The rapidity of luteinization in these ovaries and the possibility that this tissue might be elaborating a hormone that prepared the mammary glands for the hypophyseal stimulus indicated the desirability of a further test.

TABLE 2

Guinea pigs deprived of their grafts prior to injection with the pituitary extracts

NUMBER AND TYPE OF ANIMAL	NIPPLE HEIGHT	AGE OF GRAFT	AMOUNT AND PERIOD OF INJECTION	FIRST APPEARANCE OF MILK FOLLOWING THE INITIAL INJECTION	DEGREE OF LACTATION
	mm.	*months*			
Cr. 664	8	3	0.5 cc. 6 times in 4 days	56 hours	Good
Cr. 401	7	12	1.0 cc. 9 times in 5 days	Only colostrum*	
Cr. 666	4	2.5	1.0 cc. 15 times in 9 days	48 hours	Fair
Ca. 472	9	13	1.0 cc. 8 times in 5 days	36 hours	Good
Ca. 597	8	6	1.0 cc. 8 times in 5 days	36 hours	Good

* Nipple record showed marked regression.

In an effort to eliminate all possibility of a lutein influence exerted by this induced lutein tissue on the mammary glands the grafts were removed from a number of animals prior to the administration of the pituitary extract. Table 2 relates to these cases.

Among these cases guinea pigs 401 and 666 gave a rather indefinite response. An examination of the nipple record showed that considerable regression had occurred and a study of the recovered grafts and glands substantiated this evidence as the ovaries had undergone degeneration and the glands were poorly developed. It has been previously shown by Nelson and Pfiffner (1931) that, in the female guinea pig, the capacity of the mammary glands to respond to the hypophyseal hormone is markedly lessened within a few weeks following ovariotomy. In the remaining cases the mammary response was of the same order as that obtained in the animals permitted to retain their grafts during the period of pituitary treatment. In as much as histological study of the ovarian grafts in these animals showed no organized corpora lutea it would seem that the internal secretion of the corpus luteum is not an essential factor in the preparation of the mammary glands for the hypophyseal stimulus.

However, in order that all possibility of a lutein influence might be eliminated the mammary glands of four experimental cryptorchid male guinea pigs were developed by direct oestrin administration instead of through the agency of ovarian grafts. At best the parenteral administration of hormones fails to duplicate physiological conditions, but by the present procedure we hoped to simulate, in some degree, the normal development of the mammary glands in the female animal. During the first four weeks of oestrin treatment the dose was 1 R.U. daily. This was increased, successively, to 2 R.U. daily for two weeks, 4 R.U. daily for two weeks, and 12.5 R.U. daily for the final three weeks. The total amount injected in each animal was 374.5 R.U.

The nipples and mammary glands showed a very marked response and approximately 36 hours following discontinuance of oestrin and the initiation of pituitary treatment a few drops of milk could be expressed. This gradually increased to a copious flow within a few days. In so far as we could ascertain there was no difference in the secretory response of these animals as compared to those whose mammary glands had been developed under the influence of ovarian grafts. As the oestrin employed in this experiment was free of the lutein hormone the result demonstrates that lactation may be induced, when the proper stimulus is supplied, in male guinea pigs whose mammary glands have been developed under the influence of oestrin alone. The lutein hormone is not an essential factor, at least when the oestrin-induced growth is gradual and prolonged.

Discussion. The use of male animals bearing ovarian grafts in the study of mammary growth was instituted by Steinach (1912). This author as well as Athias (1915), Sand (1918), and Lipschütz (1926) observed excellent mammary growth and in some cases reported milk secretion. Moore (1921) failed to obtain lactation although he was able to demonstrate marked glandular proliferation. Although a large number of male guinea pigs with functional ovarian grafts have been examined in this laboratory only two have been observed which gave any indication of milk secretion. By far the majority of our observations indicate that while ovarian grafts in male guinea pigs are able to stimulate greater mammary growth than is ever seen in non-pregnant females they do not induce secretory activity. Although the development is sufficient to permit lactation there appears to be a lack or an insufficiency of the factor which stimulates secretion in the parturient female. We believe this factor to be an anterior pituitary principle.

The possibility exists that where milk secretion has been observed in animals carrying ovarian grafts some factor or combination of factors has served to stimulate the elaboration or release of the lactation-inducing substance in the anterior pituitary. This suggestion gains some support through the fact that in the male guinea pig the hypophyseal-ovarian rela-

tionship and its expression on the mammary glands differ, at least in a quantitative sense, from the equivalent relations in the female. It seems probable that this condition is an expression of the higher hormonal content generally considered to be present in the male hypophysis (see also Smelser 1933).

The secretion of milk which has been reported in male guinea pigs may be due to some fluctuation in this strong hypophyseal-ovarian relationship. Such a fluctuation conceivably might be occasioned by an abrupt degeneration of the ovarian graft. The cessation of an oestrin influence on the hypophysis, acting in the reciprocal manner postulated by Moore and Price (1932) for the relation between oestrin and the gonad-stimulating hormone, would engender the production or release of the lactation-inducing factor. That gonadectomy causes an increase in the gonad-stimulating content of the guinea pig hypophysis has been shown by Severinghaus (1932). Furthermore, the above concept could well be applied to the phenomenon of lactation in the parturient female since it is well known that the high oestrin content during pregnancy falls rapidly at the time of parturition. The hypothesis that removal of the ovarian influence on the hypophysis in males whose mammary glands have been proliferated through the agency of ovarian grafts will induce lactation is being investigated at the present time. A few preliminary experiments involving the removal of the ovarian grafts in otherwise untreated males are in support of this concept. It may be said, too, that other experiments in progress at the present time support the idea of an ovary-pituitary relationship in the control of lactation.

The positive character of the anterior pituitary relation to the induction of lactation is apparent from a consideration of the uniform results following its administration. Furthermore, its complete independence of the ovary, in the sense of the ovary acting as an intermediary, is shown by the occurrence of secretion irrespective of the presence or absence of the ovaries at the time of treatment. That it does depend upon ovarian influences for the development and maintenance of the mammary glands has been shown by Nelson and Pfiffner (1931). Additional evidence in this regard is offered by a few of the cases reported here (guinea pigs 401 and 666).

Although there can be no question concerning the necessity of ovarian influences on the mammary glands prior to the lactation-inducing stimulus of the anterior pituitary, it by no means has been shown definitely just what are the relative rôles played by the two known ovarian hormones. That oestrin in some form is an essential factor seems beyond question, but the importance of the lutein hormone has remained an open problem. It was with the hope of furnishing further evidence on this problem that the present experiments were undertaken. The male guinea pig carrying a

functional ovarian graft provides a test animal that has certain distinct advantages since in our experience ovarian grafts which have resided for several months in otherwise untreated male animals never contain corpora lutea, although similar grafts in females do so regularly. The reason for this phenomenon is not clear, but it seems probable that it may reside in a fundamental difference between the male and female hypophyses. Despite the absence of corpora lutea in ovaries grafted in males, it was shown that lactation could be induced with equal facility in either sex when the lactation stimulus was supplied. Furthermore, male guinea pigs which had been injected with pyramided amounts of lutein-free oestrin over an eleven week period lactated copiously when the anterior lobe hormone was administered.

Although the results of the various experiments are strong evidence that lactation may be induced in the male guinea pig in the absence of a lutein influence, they do not rule out the possibility that the corpus luteum is concerned in the mammary growth and function of normal pregnancy.

The modified psycho-sexual behavior of male animals subjected to the so-called "feminizing" influence of ovarian grafts has been given a great deal of attention by Steinach, Lipschütz, and others. These investigators state that such males assume many feminine characteristics, among them being the willingness to suckle young pigs. On the other hand, Moore (1921) observed no maternal inclinations on the part of such males. During the course of the present investigation we placed a number of lactating males with young animals removed from their mothers a few days after birth. Although these young guinea pigs made repeated and vigorous attempts to suckle they were consistently repelled by the male. When he was held forcibly the young did nurse, but not even passive resignation was exhibited during several days of enforced suckling. When it is rememberd that these males had been castrated a few days after birth, had carried functional ovarian grafts for several months, and were lactating copiously, yet showed no evidence of maternal tendencies, it seems apparent that these "feminizing" influences have induced no changes in the male psycho-sexual behavior.

The lactation response evinced by experimental cryptorchid males was of the same order as that obtained in castrated males. This demonstrates that the presence of the male hormone is not antagonistic to the action of the lactation-inducing principle upon the mammary glands, and, in this regard, confirms the related experiments of Nelson and Pfiffner (1931).

SUMMARY

Castrate and experimental cryptorchid male guinea pigs which had carried functional ovarian grafts for periods of time ranging from three to ten months have been induced to lactate by the administration of an

extract of the anterior hypophysis. This reaction was induced with equal facility in cases where the graft was removed prior to the initiation of treatment and where it was permitted to remain *in situ* during the course of hormonal treatment.

Other experimental cryptorchid animals were injected with pyramided amounts of oestrin for eleven weeks. When this treatment was followed by pituitary administration, lactation resulted.

It is concluded that, in the male guinea pig, lactation may be induced in the absence of any luteal influence operating on the mammary glands. The idea is advanced that normal lactation and some cases of experimental lactation may be explained on the basis of a release of the lactation-inducing factor by the removal of the ovarian influence on the anterior hypophysis.

BIBLIOGRAPHY

ATHIAS, M. 1915. Compt. Rend. Soc. de Biol., lxxviii, 410.
CORNER, G. W. 1930. This Journal, xcv, 43.
LIPSCHÜTZ, A., M. TIITSO, D. SVIKUL AND S. VESNJAKOV. 1926. Pflüger's Arch., ccxi, 279.
MOORE, C. R. 1921. Journ. Exp. Zoöl., xxxiii, 365.
MOORE, C. R. AND D. PRICE. 1932. Amer. Journ. Anat., l, 13.
NELSON, W. O. AND J. J. PFIFFNER. 1930. Proc. Soc. Exp. Biol. and Med., xxviii, 1.
1931. Anat. Rec., li, 51.
SAND, K. 1918. Pflüger's Arch., clxxiii, 1.
SEVERINGHAUS, A. E. 1932. This Journal, ci, 309.
SMELSER, G. K. 1933. Physiol. Zoöl., vi (in press).
STEINACH, E. 1912. Pflüger's Arch., cxliv, 71.
STRICKER, P. AND F. GRÜTER. 1929. Press Med., xxxvii, 1268.

QUANTITATIVE INVESTIGATIONS ON THE INFLUENCE OF HORMONES ON ABSORPTION[1]

Internal Secretions and Permeability II

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In a preceding paper of this series by E. Gellhorn and H. Gellhorn and in the book of the senior author on permeability, the literature on the question on the influence of hormones on permeability was reviewed and it was shown to contain a great many contradictions. Therefore it seemed desirable to investigate the question under as simple conditions as possible where changes in circulation, etc., which complicate the interpretation of data obtained by other workers could not take place. In the earlier work the frog's skin and the abdominal muscles were used as osmometers and the permeability to glucose with and without the presence of thyroxin, adrenalin and insulin was studied. The result was an increase in permeability of both membranes in solutions containing thyroxin, insulin and high concentrations of adrenalin (1:1,000,000 or more) and a decrease in lower adrenalin concentrations.

The work on thyroxin was confirmed by Saito who showed an increase in permeability of frog's skin to dyestuffs in the presence of thyroxin. He also found a greater permeability of frog's skin to dyestuffs if the frog had been fed previously with thyroid gland. More conflicting are the results of various workers in respect to the effect of insulin. Hamburger repeated our experiments with erythrocytes and muscle membranes using glucose solutions of 0.1 per cent, and obtained only negative results. He therefore believes that our results were due to too high sugar concentrations (isotonic or nearly so). On the other hand observations of Siegel on the distribution of sugar between blood and muscle seem to point to an increase in permeability by insulin. The fact that in our experiments the muscle membranes remained irritable at the end of the experiment and that in the skin membranes the unidirectional permeability was preserved seems to be in favor of a physiological significance of our results. But a renewed study of the question with an entirely different method seemed to be

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necessary for the following reasons: 1. In surviving preparations such as the muscle and skin membrane some cells may have become non-irritable without greatly affecting the irritability as indicated by the faradic currents. 2. Since frequently different cells studied under similar conditions behave differently, it is desirable to study the effect of internal secretions on permeability in another preparation not previously used for this purpose. Such a preparation should fulfill the following requirements: 1. The conditions must be simpler than in the intact organism where in the study of absorption, sugar distribution and similar processes, circulatory changes and nervous influences may bring about results similar to those to be expected if cellular permeability changes were to occur. 2. The cells are to be kept under as physiological conditions as possible to prevent irreversible permeability changes due to the gradual loss in irritability.

After a number of preliminary experiments a method described by Mond was adopted with slight modifications and fulfilled the requirements stated above in a very satisfactory manner.

METHOD. Pithed frogs were used. For perfusion of the capillaries of the gut a cannula was introduced into the coeliac artery the gastric branches of which were tied off, preventing perfusion through the stomach and loss of fluid. Through this cannula, by means of a three-way cock, either Ringer's solution or Ringer's solution containing the substance under investigation could be perfused. The liquid, after passing through the gut capillaries, was collected from an outlet cannula introduced into the portal vein.

The lumen of the gut was perfused with 3.15 per cent glucose solution through a cannula introduced just posterior to the pylorus, the outlet cannula being just anterior to the rectum.

The liquid perfused through the capillaries was divided into samples each of which represented ten minutes of perfusion. These samples were analyzed for sugar by the method of Folin-Wu.

RESULTS. 1. *Control experiments.* In order to be certain that changes in the absorption rate, which was used as the indication of permeability, were due to the administration of the substance under investigation, the perfusion rate was kept constant, the perfusing solutions were well oxygenated, and the first few samples were discarded. In many preparations hardly any adjustment was necessary to keep the perfusion constant; in others, due to marked activity of the gut musculature, rather large variations in the perfusion rate occurred. In such cases the pressure was adjusted accordingly. Small variations in the perfusion rate are unavoidable but of no significance since variations brought about by the addition of various hormones are far beyond those observed in control experiments. Furthermore, the perfusion was started and finished with Ringer's solution and between those periods Ringer plus hormone was used. Complete

reversibility of the effects of the hormone added to Ringer's solution was observed in most cases and therefore no difficulties were involved in interpreting these data. In other experiments in which, due to high concentrations of the hormones, irreversible effects were obtained, the interpretation of the results could be based on the general course of sugar absorption obtained in control experiments and others in which hormones in subliminal concentrations had been added. It may be emphasized that although in numerous experiments the sugar absorption remained almost equal over a period of two hours, not infrequently a steady decrease in sugar absorption was observed, although all conditions were kept as constant as possible. This decrease was most marked in the first periods of the experiments. It is very remarkable and indicates that blood vessels and gut are kept under physiological conditions in this preparation, since in no case a spontaneous increase in sugar absorption was observed in the control experiments, injury being invariably accompanied by an increase in permeability. (Compare Gellhorn, 1929, p. 195.)

Adrenalin. In a first group of experiments the effect of adrenalin in concentrations of from 1:500,000 to 1:25,000,000 was studied. It was found that adrenalin 1:500,000 to 1:5,000,000 considerably increased sugar absorption (fig. 1). It is of interest to note that frequently, as in the curves of figure 1, the effect of adrenalin was greater during the second application. The changes in absorption were completely reversible. Occasionally it was found that the effect was somewhat delayed, showing the maximum absorption during the perfusion periods following the adrenalin period. In lower concentrations (1:10,000,000) a decrease in absorption occurred (fig. 2). In still lower concentrations (1:25,000,000) the effects were weak. Two characteristic examples are given in figure 3, which show that during the first application a slight decrease in absorption was obtained, whereas in the second perfusion period with adrenalin the sugar absorption was slightly increased. This again seems to indicate that the adrenalin effectiveness is increased during the second period of its application. In our studies not infrequently effects of this type were observed which may be taken as an expression of a cumulative effect upon the cells which allow the sugar to permeate through the gut.

Summarizing the results, it may be said that adrenalin in relatively high concentration (1:500,000–1:5,000,000) increases, in low concentrations (1:10,000,000–1:25,000,000) decreases absorption, the effects being rather slight at 1:25,000,000. The question arises whether the effects on absorption are dependent upon the changes in the diameter of the blood vessels brought about by adrenalin.

The results of our experiments show conclusively that no relation exists between the vasoconstrictor effect of adrenalin and its influence on sugar absorption. In concentrations of 1:500,000 to 1:1,000,000 the constric-

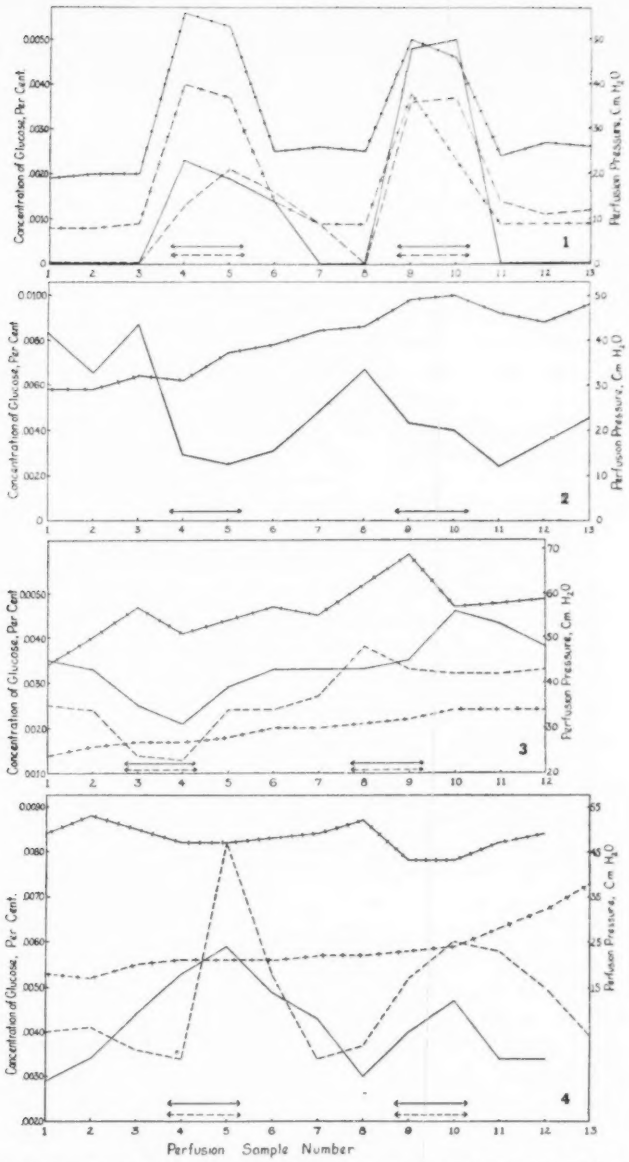

Fig. 1. Experiment A (solid line) and experiment B (broken line) with adrenalin 1:1,000,000, which was administered during the collection of the samples covered by the arrows. Other samples, perfusion with Ringer's solution.

The solid crossed line shows perfusion pressures for the experiment A, depicted by the solid line; the broken crossed line perfusion pressures for the experiment, B, depicted by the broken line.

Fig. 2. Experiment with adrenalin 1:10,000,000. Details as in figure 1.

Fig. 3. Experiment A (solid line) and experiment B (broken line) with adrenalin 1:25,000,000.

Fig. 4. Experiment A (solid line) and experiment B (broken line) with adrenalin 1:50,000,000 plus serum 1:5,000. Details as in figure 1.

tive effect was very marked and accompanied by an increase in absorption. In lower concentrations (compare the curve in fig. 2) not the slightest constrictive effect was observed in spite of very considerable changes in sugar absorption. As was mentioned above, any constrictive effect was at once compensated in order to keep the perfusion rate constant. It may therefore be said that independent of its vascular effects adrenalin displays specific effects on the absorption from the gut which depend on the concentration and consist either of an increase or a decrease in absorption.

Abderhalden and Gellhorn showed in 1923 that in the presence of small amounts of serum the effectiveness of adrenalin upon the heart is greatly increased. The threshold for the positive inotropic action is lowered and the duration of the adrenalin effect is increased. Therefore the question

TABLE 1

Experiment to determine the effect of serum on sugar absorption

CAPILLARIES PERFUSED WITH:	PERFUSION SAMPLE NUMBER	CONCENTRATION OF GLUCOSE
		per cent
Ringer	1	0.0028
Ringer	2	0.0025
Ringer plus serum, 1:500	3	0.0018
Ringer plus serum, 1:500	4	0.0020
Ringer	5	0.0018
Ringer	6	0.0014
Ringer	7	0.0014
Ringer plus serum, 1:500	8	0.0014
Ringer plus serum, 1:500	9	0.0016
Ringer	10	0.0015
Ringer	11	0.0016
Ringer	12	0.0016

was investigated whether the action which adrenalin has on absorption may also be enhanced by serum. In our experiments frog serum was used in concentration of 1:500 or 1:5,000 diluted with Ringer's solutions. As table 1 indicates, frog's serum 1:500 in Ringer alone is without influence on sugar absorption.

Numerous experiments performed with the addition of serum to adrenalin-Ringer in the concentrations mentioned above proved conclusively that adrenalin in such solutions is more effective in influencing sugar absorption than in pure Ringer's solution. No significant differences exist between the two serum concentrations. In concentrations of 1:25,000,000 adrenalin a distinct and reversible decrease in sugar absorption regularly occurs. As far as the vasoconstrictor effect is concerned, the results vary somewhat, since in some instances vasoconstriction was observed, and in others it was

absent. Although the regular decrease in sugar absorption caused by adrenalin 1:25,000,000 in the presence of serum strongly suggests an increased effectiveness of adrenalin, the experiments shown in figure 4 prove it beyond doubt. In these cases adrenalin was effective even in a concentration of 1:50,000,000 and increased sugar absorption reversibly. Such action in the absence of serum requires a concentration of adrenalin of at least 1:5,000,000. These marked effects of adrenalin on absorption were not accompanied by vasoconstriction, as is shown in the figure.

Thyroxin. The experiments with thyroxin were carried out in concentrations of from 1:50,000 to 1:200,000. The pH of Ringer's solution with and without thyroxin was adjusted to the same value (pH = 7.6). The effect in concentrations of 1:50,000 was regularly a marked increase in the absorption of sugar which frequently was more or less irreversible, as shown in experiment A in figure 5. This interpretation is justified, since, as was already emphasized, a spontaneous increase in sugar absorption never occurred. Occasionally this increase was delayed, as experiment A in figure 5 indicates. As was shown in the work with adrenalin, an increase in sensitivity to the same drug during its second application was also observed with thyroxin, but to a much greater extent. In fact, it was this group of experiments which called our attention to this phenomenon. Figure 6 gives an example. In both cases the thyroxin effect is very marked during the second application, while it is either completely or almost absent during the first. One also obtains the impression from these experiments that the speed with which the reaction is brought about is greater during the second than during the first application of the drug. That is particularly distinct in experiment A, in which the increase in sugar absorption occurred with very great delay during the first part of the experiment but much faster in the second period, although even here after the perfusion with thyroxin.

It is significant that thyroxin never influenced the perfusion rate. In the concentrations mentioned above it was without influence on the capillaries, causing neither contraction nor dilatation. It is assumed that thyroxin increases the permeability of the gut cells adjacent to the capillaries, but whether it also has a specific influence on the permeability of capillaries remains undecided.

In concentrations of 1:100,000 an increase in sugar absorption was also observed. In this concentration there was again a characteristic increase in sensitivity in the second application of thyroxin. Frequently the first application was without effect, while the second caused a marked increase in sugar absorption. This increase was characteristically delayed, occurring after the perfusion with thyroxin was over.

Only a slight increase in sugar absorption occurred in experiments with thyroxin 1:200,000. Still lower concentrations were not examined.

Another series of thyroxin experiments was performed in the presence of serum (frog's serum 1:500 in Ringer's solution). Neither the type nor the range of concentration in which thyroxin was effective was changed. Figure 7 shows two curves which illustrate the marked increase in absorp-

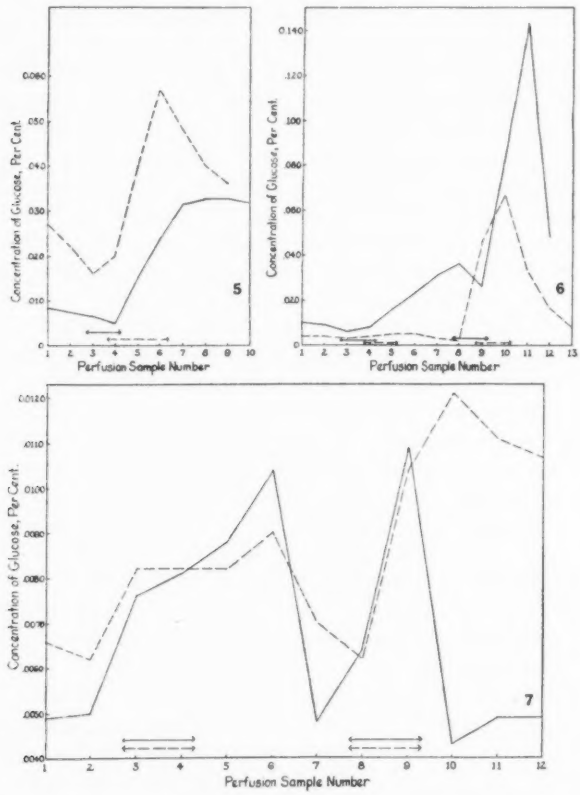

Fig. 5. Two experiments, A, solid line, and B, broken line, with thyroxin 1:50,000.
Fig. 6. Two experiments, A, solid line, and B, broken line, with thyroxin 1:50,000. Graphs of perfusion pressures not given; otherwise details are as in figure 1.
Fig. 7. Experiments with thyroxin: A, solid line, 1:50,000 with serum 1:500; B, broken line, thyroxin 1:100,000 with serum 1:500. Details as in figure 5.

tion during the thyroxin periods. One might get the impression from these curves that serum accelerated the thyroxin effect. But other experiments showed a delayed increase in sugar absorption, and in several experiments no influence of thyroxin 1:100,000 was obtained in the presence

of serum. This justifies the conclusion that no significant changes in the thyroxin effect are brought about by the simultaneous application of serum.

Insulin. A preparation from Lilly (Iletin) was used. Since it contains 0.2 per cent phenol, corresponding amounts of phenol were added to Ringer's solution so that it differed from insulin-Ringer only by its insulin content. Insulin was examined in concentrations of from 0.02 to 0.0033 unit per cubic centimeter. It was found that the insulin effects on sugar absorption are rather weak unless the pH is at least 7.4. On those experiments the following description is based. A group of experiments per-

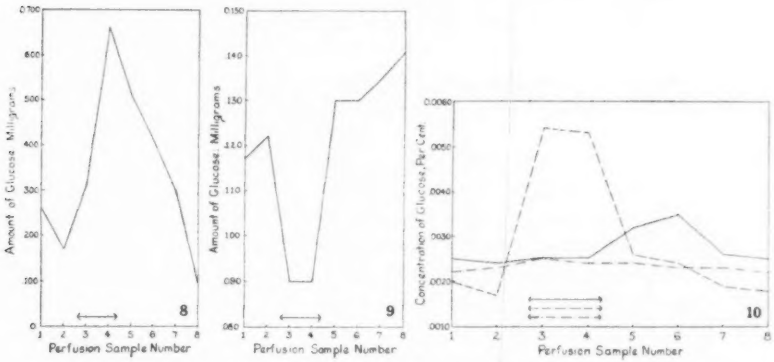

Fig. 8. Experiment with insulin, 0.01 unit per cubic centimeter. Details as in figure 5.

Fig. 9. Experiment with insulin, 0.005 unit per cubic centimeter. Details as in figure 5.

Fig. 10. Solid line: experiment with insulin, 0.02 unit per cubic centimeter with serum, 1:500.

Broken line: experiment with insulin, 0.0067 unit per cubic centimeter with serum, 1:500.

Dot and dash line: experiment with insulin, 0.005 unit per cubic centimeter with serum, 1:500.

Details as in figure 5.

formed at a pH of 7.2 is omitted. Figure 8 shows that insulin in a concentration of 0.01 unit per cubic centimeter increases absorption. With 0.02 unit per cubic centimeter the effect is the same, but it is frequently irreversible. Occasionally a delayed effect was observed: i.e., the greatest increase in sugar absorption occurred in the Ringer periods which followed the application of insulin. Five-thousandths unit per cubic centimeter decreased reversibly the absorption of sugar, as shown in figure 9; 0.0033 unit per cubic centimeter was without any effect. In these experiments the question was also investigated whether the addition of small amounts of serum as used in the previously described experiments had any influence on the

insulin effect. Since the strength of this effect varies somewhat between different preparations, it is sometimes difficult to decide such a question. But the comparison of the effects of insulin in 13 experiments with and 13 without serum shows undoubtedly that serum decreases the insulin effect. In concentrations of insulin of 0.03 and 0.01 unit per cubic centimeter, in which insulin regularly increases sugar absorption, this effect is frequently lacking in the presence of frog's serum. Examples of positive experiments, i.e., such ones in which an alteration of absorption occurred, are reproduced in figure 10. To those the typical curve of an experiment with 0.005 unit per cubic centimeter of insulin plus serum is added. The sugar absorption remains unchanged. A decrease in absorption was never observed in serum plus insulin mixtures. Three facts seem to indicate that the insulin effect is decreased in the presence of serum: 1. The rather irregular success of insulin plus serum experiments in contrast to those in which insulin alone was administered. 2. Even in the positive experiments the effect was usually less and often delayed. 3. The smallest concentration which occasionally gave positive results was 0.0067 unit per cubic centimeter in the presence of serum, but 0.005 unit per cubic centimeter without serum. In the latter case the absorption was regularly influenced.

As in the thyroxin experiment, the perfusion rate remained unchanged in the insulin periods. Therefore its effect on sugar absorption cannot depend on vascular effects.

Discussion. The experiments have shown that adrenalin, thyroxin, and insulin influence the sugar absorption from the gut in spite of the constancy of the perfusion rate. This proves that the permeability of the cells mainly concerned with the regulation of the absorption rate is altered. In view of the work of Mond it seems probable that the layers of the gut adjacent to the capillaries are involved. Whether the capillaries themselves are also altered is to be decided by experiments performed with a different method. The hormones under investigation exert a regular influence on the absorption from the gut in concentrations similar to those in which these substances produce well known effects on the heart, blood vessels, metabolism, etc., in frog. It may therefore be said that the effects described in this paper are equally significant. The results emphasize not only the importance of hormones for the permeability of the cell but also for the regulation of absorption under physiological and pharmacological conditions.

SUMMARY

The influence of adrenalin, thyroxin and insulin on the absorption of glucose from the gut was studied in Rana esculenta by perfusing the gut with isotonic glucose solution and by perfusing the blood vessels supplying

the gut with Ringer's with and without the hormones mentioned above. The results are as follows:

1. Adrenalin increases sugar absorption in concentrations of from 1:500,000 to 1:5,000,000, whereas it decreases absorption in concentrations of from 1:10,000,000 to 1:25,000,000. Since changes in the perfusion rate due to the vasoconstrictor effect are readily removed by pressure adjustment, the effect on absorption is independent of them. Moreover, there is no parallelism between the effects on the blood vessels and on the gut, since not infrequently striking changes in absorption occurred, although vasoconstriction or dilatation was completely lacking.

2. Thyroxin increases sugar absorption in concentrations of from 1:50,000 to 1:200,000.

3. Insulin increases sugar absorption in concentrations of from 0.02 to 0.01 unit per cubic centimeter and decreases it with a concentration of 0.005 unit per cubic centimeter.

4. Thyroxin and insulin do not cause any vascular changes.

5. The presence of small amounts of frog's serum to Ringer's solution (1:500 or 1:5,000) although without effect on blood vessels and absorption changes considerably the effect of hormones on absorption. The effectiveness of adrenalin is considerably increased, that of insulin seems to be somewhat decreased, whereas thyroxin is not influenced in its effect on absorption.

6. The experiments prove that adrenalin, thyroxin and insulin alter the permeability of the gut in a reversible fashion.

7. Not infrequently, in the smaller concentrations, the first application of the hormone to a preparation was ineffective, while the second, due probably to sensitization, increased sugar absorption.

BIBLIOGRAPHY

ABDERHALDEN, E. AND E. GELLHORN. 1923. Pflüger's Arch., cxcix, 437.
GELLHORN, E. AND H. GELLHORN. 1928. Pflüger's Arch., ccxxi, 247.
GELLHORN, E. 1929. Das Permeabilitatsproblem, seine physiologische und allgemein-pathologische Bedeutung. Berlin.
HAMBURGER, R. J. 1930. Thesis. Gröningen.
MOND, R. 1924. Pflüger's Arch., ccvi, 172.
SAITO, T. 1930. Fol. endocrin. Jap., vi, 19.
SIEGEL, R. 1929. Klin. Wochenschr., p. 1655.

ON THE PRINCIPLE OF AUTONOMIC NERVOUS ACTION

Observations on the Resistance to Temperature of the Endings of Vagus and Sympathetic in Heart

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Although we have a wealth of information as to the influence of physico-chemical factors (pH, K, Ca, etc.) of the perfusion liquid upon the effect of nervous stimulation, particularly in reference to the vagus and sympathetic endings, almost no data are known which indicate characteristic differences between the two antagonistic nerve endings if physical stimuli be employed. That physical properties may differ in nerves which differ functionally was shown first by Hafemann who found between the motor and sensory fibers of the frog's sciatic nerve a marked difference in the resistance to high temperatures. Therefore we studied the resistance of vagus and sympathetic endings in heart to heat, with a view 1, to determine how, if at all, the endings of the vagus and sympathetic in the heart differ in susceptibility to injury by heat; 2, to relate the results to the mechanism of nervous action as it is understood today on the basis of the fundamental papers of Loewi, Finkleman, Engelhardt, Hansen, and others. (Compare the reviews of Gellhorn, 1926, and Asher, 1931.)

Procedure. The preparation used in these experiments was that described by Finkleman (7). The frog (Rana esculenta) was decerebrated by cutting across just back of the eyes, and the skin and visceral organs, with the exception of the heart and liver, were removed. The heart was perfused with Ringer's solution by means of a cannula inserted through the wall of the cava posterior at the anterior border of the liver and into the sinus of the heart. The right branch of the aorta was ligated, and a cannula was introduced into the left branch to carry off the perfusing liquid. It was found advisable to flush the heart with Ringer's solution as soon as possible when making the preparation, to free the heart chambers and aorta of blood which might otherwise form clots and impair, or even stop the perfusion.

The vertebral column was transected between the third and fourth thoracic vertebra, and the heart-and-nerve preparation was pinned to a small platform mounted within a moist-chamber. The vagus was stimu-

lated through a pair of fine platinum electrodes at the cephalic end of the preparation, one being inserted within the spinal canal to the level of the medulla, while the other was in contact with the musculature of the back. The sympathetic was stimulated by means of similar electrodes at the opposite (caudal) end of the preparation, one being inserted within the spinal canal to a distance of four or five millimeters, while the other was in contact with the back at the same level. Thus, by means of a double-throw switch, either nerve could be stimulated at will. The stimulating current was provided by a single dry-cell in the primary circuit of an inductorium.

The perfusing liquid was heated by allowing it to pass through a flat coil of glass tubing which rested on an electrically-heated microscope warm-stage. Between this heating coil and the cannula a T-tube was placed in the system, and a thermometer was inserted into the free arm in such a way that the solution flowed past the bulb on its way to the heart. To make possible an exact determination of the temperature within the heart, a thermocouple was used, the warm-junction being wrapped around the bulb of the thermometer, while the cold-junction was inserted through the wall of the rubber tubing and up into the tip of the cannula, which, being inserted into the sinus, was assumed to have approximately the same temperature as the heart tissue. Each degree of difference between the temperature of the two junctions gave a deflection of three units on the scale of the galvanometer. Oxygen which had first passed through a bottle of water (to saturate it with moisture) was allowed to flow into the moist chamber, and the Ringer's solution used in the perfusion was aerated by allowing oxygen to bubble through the supply in the reservoir.

The strength of stimulus required to produce a definite effect on the rate and amplitude of the heart contractions was determined for each nerve at the beginning of the experiment. (In some of the experiments the rate and amplitude were very great at first, so that no effect of the sympathetic nerve could be detected until after the heart had been heated once.) Then the perfusing solution was heated until the heart stopped beating, the warm liquid remaining in the system was drained out by means of an outlet (T-tube) near the cannula, and as soon as the heart had cooled to room temperature and had resumed its beating, the threshold for each nerve was again determined. This heating and cooling procedure was repeated until one of the nerves failed to affect the heart even when a maximal stimulus was given. In some cases but one heating was required, while in others the heart had to be heated and cooled several times. In the course of the last few experiments it was also found that a temperature of 38 to 40 degrees would produce satisfactory results even though no standstill was obtained at this temperature; however, the higher temperature will produce the same effect more quickly.

EXPERIMENTAL RESULTS. Out of sixteen valid experiments (i.e., experiments in which the perfusion remained good throughout the investigation) eleven gave results in which the vagus nerve was clearly more greatly injured by the high temperature than was the sympathetic. Of these eleven, nine showed a total disappearance of the vagus effect while the sympathetic remained active; in the two remaining cases the vagus effect was not totally abolished, but the threshold was raised to a very high level, while the sympathetic threshold remained much lower. In one experiment both nerves survived six heatings with very little increase in the threshold, but in the light of the other experiments it seems probable that this was,

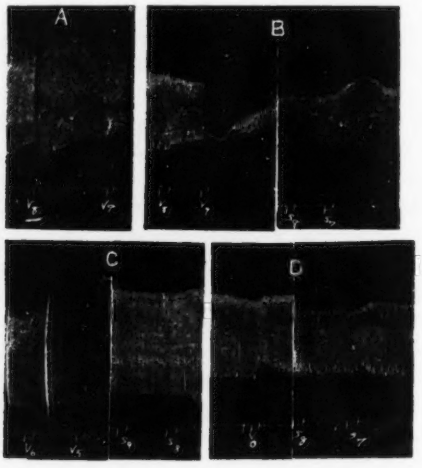

Fig. 1. Stimulation of vagus and sympathetic before and after heating. A, before heating; B, C, D after 1st, 2nd, and 3rd heating, respectively.

for some reason, an unusually resistant preparation which could eventually have shown the same results with repeated heatings.

In four of the experiments both the vagus and the sympathetic effect disappeared at the same time. But in *no* case was the sympathetic effect found to disappear before the vagus, nor did the threshold for the sympathetic ever rise to a high value while the vagus threshold remained comparatively low. In every case where a difference in the degree of injury to the two nerves was indicated, it was the vagus nerve which had suffered the most. These results are illustrated by the following experiment:

April 20. Before the heart was heated, the sympathetic threshold could not be determined because the rate and amplitude of the beat were already near the maximum. The vagus was found to completely inhibit the heart with an 8 cm. separation

of the primary and secondary coil of the inductorium, V_8. (See fig. 1 A.) The heart was then heated to a standstill (35°C.) and cooled, after which the vagus threshold was found to be V_7, and the sympathetic threshold was the same, S_7 (fig. 1 B). After the second heating (35°) the effective stimulus for the vagus had increased to V_5, while the sympathetic was effective at S_5 (fig. 1 C). After the third heating (44°) the heart was not affected by the strongest vagus stimulus which could be given, while the sympathetic stimulation was still effective at S_7 (fig. 1 D).

In many cases, after the heart had been heated several times, stimulation of the *vagus* nerve produced a typical *sympathetic* effect. In fact, it was sometimes found that, using the same strength of stimulus for the two nerves, a much greater increase in the amplitude and rate of the heart contractions resulted from the vagus stimulation than was obtained by stimulating the sympathetic nerve. To offset the possibility that such a result might cover up a weak inhibitory effect of the vagus, the sympathetic effect was eliminated in several of these experiments by perfusing the heart with a solution of ergotamine acid phosphate in Ringer's solution, in a concentration of 1:10,000. Stimulation of the vagus nerve following the disappearance of

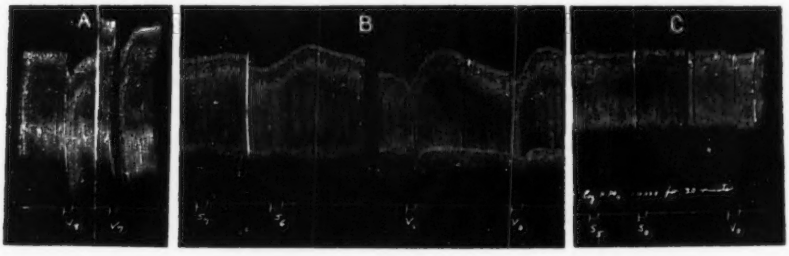

Fig. 2. As in figure 1. A, before heating; B, after 1st heating; C, after application of ergotamine, 1:10,000.

the sympathetic effect resulted in no inhibition of the heart, as is shown by the following experiment:

May 16. Here again the sympathetic threshold could not be determined at the beginning of the experiment. The effective vagus stimulus was found to be V_7. But after the temperature had been raised to 38° (without producing a standstill) and reduced again to 30° no inhibition could be produced by vagus stimulation, while a stimulus of S_7 increased the amplitude of the heart contractions slightly, and S_6 produced a marked increase. The vagus stimulation also produced a marked sympathetic effect, so the heart was perfused with a solution of ergotamine acid phosphate (1:10,000) for twenty minutes, after which no sympathetic effect could be obtained from either nerve, and the vagus was still completely ineffective (fig. 2).

The objection might be raised that the disappearance of the vagus effect in the above experiments was more a matter of natural death of the nerve than of injury by heat. The following results show conclusively that such is not the case. A preparation was made which, for some reason, failed completely to react to sympathetic stimulation. Perfusion was stopped at 10:00 p.m. and the heart was left in the moist-chamber. The following

morning at 10:00 a.m. the heart was found to be still beating rather feebly, so the perfusion was resumed, after which the contractions became gradually stronger and more regular until a normal beat was established. Then the vagus nerve was stimulated, with the result that the amplitude of the contraction was greatly reduced during and immediately following the stimulus. The perfusion was continued throughout the day, and at 4:00 p.m. vagus stimulation was found to produce complete inhibition of the heart. Thus the nerve was found to have survived 18 hours, 12 of which

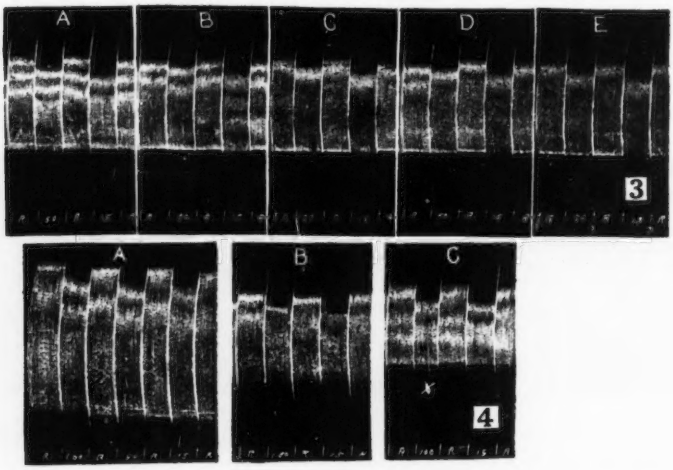

Fig. 3. Control experiment on the acetyl choline effect in heart. The different recordings (A, B, C, D, E) are made in intervals of 12 minutes. R = Ringer. The numbers 50, 15, etc., represent the acetyl choline concentration, 1:50,000,000 and 1:15,000,000, respectively.

Fig. 4. A, before heating, B and C after the 1st and the 2nd heating, respectively. In A, B, and C the effect of acetyl choline is determined at 20°. Designations as in figure 3.

the heart was without perfusion! In another experiment the threshold for the vagus remained practically unchanged, V_5, from 5:00 p.m. until 11:00 p.m., at which time the experiment was discontinued. These experiments show definitely that the vagus nerve will remain active over considerable lengths of time, and even under unfavorable conditions, at room temperature. Therefore, differences in the survival of the two nerve effects must be due to a difference in the resistance of the nerve endings to high temperatures.

The question arises as to the cause of the loss of vagus function after a temporary application of higher temperatures. On the basis of the work of

Loewi and co-workers, Engelhardt, Plattner, Finkleman, and others, it must be assumed that the cause of the vagus effect is the liberation of acetyl choline. If the vagus fails to react it may be due either to the inability of the nerve endings to release, or cause the production of, acetyl choline, or it may be due to the fact that the structure upon which the acetyl choline acts becomes unable to respond.

In order to decide between the two main possibilities as to the cause of the disappearance of the vagus effect, experiments were performed on the heart strip of Rana esculenta. Half of the ventricle was cut away by two incisions: one between auricle and ventricle, and another at right angles to it. The ventricle strip with auricle and sinus still attached was suspended in oxygenated Ringer's solution. The automatic contractions of the heart strip were recorded with an isotonic lever. Before the question was

TABLE 1

The influence of acetyl choline on the frequency of a heart strip before and after different heating periods

	a.c.* 1:15,000,000	R**	a.c. 1:5,000,000	R	a.c. 1:2,000,000	R
Before heating	25‡	27	23	27	12	27
After 1st heating	24	32	18	31	12	30
After 2nd heating	24	30	17	30	24	28
After 3rd heating	22	30	12§	28	9§	28
After 4th heating	22	30	12§	28	9§	28

* a.c. = acetyl choline.

** Ringer's solution.

‡ The numbers indicate beats per minute

§ Disturbance of the conduction of heart impulse (Luciani periods, etc.)

examined whether the heart remains able to respond to acetyl choline after several heating periods control experiments were performed. The heart strip was tested with acetyl choline a number of times at room temperature during a period equal to that used in the heating experiments. As figure 3 indicates, the susceptibility of the heart strip to acetyl choline remains unchanged. Very weak concentrations of acetyl choline (1:50,000,000) still produce a reversible inotropic effect. Then several experiments were performed in which before and after several heating periods the acetyl choline effect was determined. Figure 4 may serve as an example indicating that the effect of acetyl choline on the heart is increased even after the temporary application of heat which regularly leads to a decrease, or complete loss of the vagus effect. Perhaps still more striking is the experiment (compare table 1) in which the negative chronotropic effect of acetyl choline before and after several heating periods is

reproduced. All acetyl choline tests of this experiment were performed at 20°. In the first two heatings the temperature was raised to 40°, in the last two even to 42°, so that we can be absolutely sure that under these conditions the vagus effect was abolished. Nevertheless the negative inotropic effect of acetyl choline was even more marked, since in higher concentrations (1:2,000,000 and 1:5,000,000) disturbances in the conduction of the heart impulse (halving of the rhythm, Luciani's periods) occurred which were absent before the heating and even after the first heating period. The experiments prove that both the negative inotropic and the negative chronotropic effect are increased after several heating periods. Therefore it may be said that *the abolition of the vagus effect by heat is due to a disturbance of the production or release of the acetyl choline and certainly not to a loss of susceptibility of the heart to it.*

The recent work of Finkleman, Cannon, Lanz and Asher (1932) make it highly probable that the sympathetic effect is due to the liberation of adrenalin. In our experiments the sympathetic effect was either of the same strength after several heatings as before or slightly less (requiring a somewhat stronger current to evoke the typical sympathetic changes in the heart). It was possible that the relative constancy of the sympathetic effect was due to an increased susceptibility of the heart to adrenalin and that the liberation of adrenalin was impaired, as is the case with acetyl choline. Therefore experiments were performed in which the adrenalin effect was studied before and after several heatings. The result is that the reaction of the heart strip to adrenalin is unaltered by several heating periods. It must be assumed that the process connected with the liberation of acetyl choline is much more sensitive to heat than is the liberation of adrenalin and that this is the cause of the specific effects described in this paper. In this respect it may be of interest to note that Plattner and Galehr gave numerous proofs of the instability of acetyl choline under physiological conditions. As to the stability of the endings of the sympathetic, it may be that it is related to the fact that adrenalin increases the resistance of the heart to heat and increases the upper temperature limit at which spontaneous automaticity is observed (Gellhorn, 1924).

SUMMARY

A comparative study of the susceptibility of the vagus and sympathetic endings in heart (Rana esculenta) to heat is made with the result that after one or more heatings to 40°–42° the vagus ceases to react, although the sympathetic endings are either unchanged or show only a slightly higher threshold.

A heart strip subjected to several heatings which abolish the reaction of the vagus endings shows either no change in its susceptibility to acetyl choline or a greater susceptibility. The effect of adrenalin on the heart

remains unaltered by several heatings. Therefore it is concluded that the abolition of the vagus effect by heat is due to a disturbance of the production or relase of acetyl choline and not to a loss of susceptibility of the heart to it.

BIBLIOGRAPHY

ASHER, L. 1931. In GELLHORN's Lehrbuch der allgemeinen Physiologie. Leipzig.
ASHER, L. 1932. Klin. Wochenschr., xi, 1292.
CANNON, W. B. 1931. Endocrinol., xv, 473.
ENGELHARDT, E. 1931. Pflüger's Arch., ccxxvii, 220.
FINKLEMAN, B. 1928. Journ. Physiol., lxv, p. xxx.
1930. Journ. Physiol., lxx, 145.
GALEHR, O. UND PLATTNER. 1928. Pflüger's Arch., ccxviii, 488, 506.
GELLHORN, E. 1924. Pflüger's Arch., cciii, 141.
1926. Neuere Ergebnisse der Physiologie. Leipzig.
HAFEMANN, M. 1908. Pflüger's Arch., cxxii, 1.
HANSEN, K. AND RECH. 1932. Zeitschr. f. Biol., xcii, 191.
LANZ, A. B. 1928. Arch. neerland. de physiol., xii, 433, xiii, 423.
LOEWI, O. 1921–24. Pflüger's Arch., clxxxix, 239; cxciii, 201; cciii, 361, 641.
PLATTNER, F. AND BAUER. 1928. Pflüger's Arch., ccxx, 180.
PLATTNER, F. AND HINTNER. 1929. Pflüger's Arch., ccxxii, 395.

THE RELATIVE IMPORTANCE OF THE PERFORMANCE OF WORK AND THE INITIAL FIBER LENGTH IN DETERMINING THE MAGNITUDE OF ENERGY LIBERATION IN THE HEART

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The mechanism of the regulation of energy liberation by the heart under varying conditions of load is a problem of primary importance both in the pure and applied physiology of the heart. It was observed by Knowlton and Starling (1912) that the heart compensated for an increase in load by an increase in the diastolic fiber length. This occurs automatically, because when the heart is unable to eject its full complement of blood, there is a residual quantity which remains and supplements that entering the heart during diastole, bringing about an effective increase in volume at the end of diastole over that which existed before the increased work was imposed. Starling (1915) based his original law of the heart upon this observation. Rhode (1912) was the first to observe that changes in the load on the heart affected its oxygen consumption, using the heart of the cat and rabbit perfused with Ringer's fluid. Evans (1914), using the heart-lung preparation, made a similar observation. Lüscher (1920), employing the frog's ventricle, found increased oxygen consumption with increased work and tried to correlate the energy liberation with the pressure-volume product. In this he was unsuccessful. None of the foregoing workers had been able to satisfactorily correlate the energy liberation with any measurable variable or set of variables.

Starling and Visscher (1927) were able to show in the dog's heart-lung that over the ranges of pressure and output which they used, which covered the whole of the physiological range, there was a very simple and invariably uniform relation between the energy liberation and the diastolic volume of the ventricles. These workers inferred that the factors affecting the energy liberation during contraction were operative during a very short period of time at the onset of contraction, perhaps during the isometric phase, and that subsequent events, particularly the extent to which the heart was emptied, was a matter of small consequence to the determination of energy liberation during the contraction. Hemingway and Fee (1927) were able to corroborate the results of Starling and Visscher, using a slightly dif-

ferent method, but employing the heart-lung preparation. Clark and White (1928) and Eismayer and Quincke (1930), using the frog's heart, likewise found a direct and invariable relation between diastolic fiber length and energy liberation in contraction.

Stella (1931) has recently investigated this problem by a new method on the turtle's ventricle and has come to a contrary conclusion, namely, that the energy liberation is a function not only of initial fiber length, but of the work which the fiber does during contraction. He attempted to correlate his observations with the "Fenn effect" observed in striated muscle. Fenn (1923) has shown that striated muscle liberates more energy in contraction when it does work than when contracting isometrically. It appears, moreover, that the "extra energy" is roughly proportional to the work done. In nearly unloaded isotonic or auxotonic contraction the energy output is less than under more nearly optimal conditions of loading. Stella compared very light loads with heavier loadings at a single diastolic volume with respect to energy liberation. A proper testing of the "Fenn effect" in cardiac muscle should consist in comparison of optimal loading with still heavier loading approaching an isometric contraction. This is particularly so because according to Starling and Visscher the regulation of energy output in cardiac muscle is a function of fiber length, and if a contraction in which much shortening occurs liberates less energy than one in which less shortening occurs, it must still be proven that in the first case the smaller amount of energy liberation was not due to a more extended influence of fiber length throughout the contraction period than Starling and Visscher found in the mammalian heart. It must be noted that Starling and Visscher worked at temperatures of 35° to 37°C. Stella employed low temperatures. The duration of energy liberation is greatly prolonged at low temperatures and, whereas in the mammalian heart it was found that events occurring subsequent to the isometric phase in contraction were irrelevant to the magnitude of energy liberation, it is by no means certain that at 10°C. the same situation would be found to exist. Consequently a satisfactory test of the occurrence or non-occurrence of the "Fenn effect" must be made under the opposite conditions mentioned above. We have therefore studied the oxygen consumption under such conditions of loading that an increase in the arterial pressure against which the heart is made to pump begins to limit the cardiac output to such an extent that the work done diminishes. Under such conditions the diastolic volume can be kept constant and the systolic volume is greater in the case where less work is done than where more is accomplished, thus establishing the conditions for a crucial test.

METHOD. The apparatus shown in diagrammatic form in figure 1 has been used to measure oxygen consumption. In principle it is similar to that employed by Stella (1931), but embodies certain important improvements which we have found

essential. In order to measure diastolic volume accurately under all conditions, we have found it necessary to convert the chamber around the heart into a closed cardiometer, filling it with pure nitrogen to avoid error in oxygen consumption measurements due to absorption of oxygen from the exterior. It is then necessary to measure the fluid which filters through the heart in determining oxygen usage. This is accomplished by calibrating a tube joined to the cardiometer vessel *B*, and

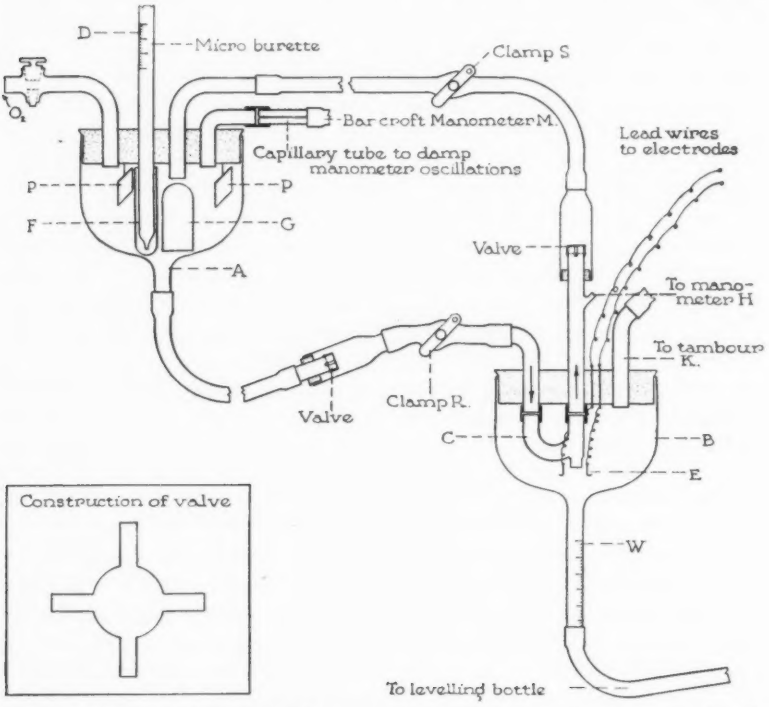

Fig. 1. Diagram of apparatus for measurement of oxygen consumption and work of isolated ventricle.

reading the difference in level of fluid in it at the beginning and end of a period of observation, subtracting the figure from the apparent oxygen consumption.

In Stella's method the chamber containing the heart is converted into a cardiometer by clamping the tube connecting to the venous reservoir. As may be seen by reference to his diagram, the clamp must be closed at the end of a complete systole if the volumes derived from the cardiometer tracing are to represent true diastolic volumes. At low arterial resistances it may be assumed that at each systole the heart is completely emptied and one can obtain an accurate measure of the heart volume in diastole. At higher arterial resistances, however, where stroke-output decreases, the systolic contraction does not empty the heart. Consequently, an error is introduced increasing in magnitude as the arterial pressure increases.

The constant filtration of fluid through the heart in appreciable amounts makes a permanent base line of no significance and consequently it cannot be used as a point of reference. It is therefore necessary to determine the absolute systolic volume each time the diastolic volume is to be measured.

The heart cannula described by Stella had too large a "dead space," which is reduced to 0.1 cc. in the form *C* which we employed. By shortening the "common arm" of the cannula practically the whole of the output of the heart is exchanged for fresh fluid at each beat, whereas with a long common arm much or most of the contraction is wasted as far as renewing the fluid in the heart is concerned. The valves employed permitted a large volume to flow at a low pressure. A piece of thin rubber cut in the shape indicated in the insert in figure 1 was fitted on the end of a perforated rubber stopper, and was held in place by a thin rubber ring to serve as the valve.

The venous pressure is altered by raising or lowering the venous chamber *A*. The peripheral resistance is varied by a screw clamp at *S*. As the blood enters chamber *A* it flows over a perforated inverted test tube tip *G*, affording adequate surface for gaseous interchange. The CO_2 produced is absorbed by 30 per cent KOH on pieces of porous porcelain, *P*, suspended in the venous chamber. Fresh alkali was used for each experiment.

Tube *M* leads to one side of a Barcroft differential manometer, the other side of which is connected to a compensating bottle of a volume equal to the air space in the venous reservoir. As oxygen was utilized by the heart, the volume of air in the chamber diminished, as is indicated by the manometer. By gradually adding fluid from the burette, *D*, the manometer oscillations were kept at the same point. This kept the pressure constant, and prevented changes in diastolic filling which otherwise occur due to the change in air pressure in the venous reservoir. The fluid added entered tube *F*, compensating for the volume of oxygen used, but not changing the amount of circulating fluid.

The side tube *H* leads to a Hürthle manometer which records arterial pressure. Tube *K* is connected to a Marey tambour which was used to measure ventricular volume. At the end of each determination the venous inflow was clamped off and the arterial resistance lowered. The resulting complete emptying of the heart enabled the recording of both stroke output and diastolic volume for each set of conditions. Both recording instruments were calibrated at the end of each experiment.

Turtles approximately six inches in length were used for the experiments, which extended from March through June. They were bled from the aortic arch, and the blood defibrinated by whipping. This defibrinated blood was added to five or ten times its volume of Ringer's solution to make up the perfusion fluid. After opening the auricles and cutting the interauricular septum and a-v valves the cannula was tied into the ventricle by a ligature around the a-v groove. By short lengths of stiff rubber tubing the cannula was attached to the venous and arterial tubes of the artificial circulation. By means of platinum electrodes, *E*, the heart was stimulated by a break shock twelve times a minute. The standard time for each determination was twenty minutes. Oxygen was allowed to flow through chamber *A*, and the cardiometer was filled with nitrogen. The apparatus was then immersed in a 300 liter water bath maintained at 24°C., constant during a single determination to within a few thousandths of a degree. Thirty minutes were allowed for the gases and perfusion fluid to come to thermal and diffusion equilibrium before observations were begun.

Between each type of observation the heart was allowed to work under the new

conditions of venous inflow and arterial resistance for 10 to 15 minutes, to reach a steady state. An initial record of the pressure and volume changes was then made. At the end of the period employed, usually twenty minutes, the burette reading of oxygen consumption was recorded, and another pressure and diastolic volume record made. After recording the movements of the tambour under the conditions of filling used during the observation, the venous tube was clamped at R, the clamp S released, and the venous reservoir lowered, so as to enable the heart to empty completely, in order to obtain the absolute systolic volume and thus be able to measure the true diastolic volume during the period of observation. The volume of fluid which filtered through the heart during the period of the determination was measured on the calibrated tube W and subtracted from the burette reading to give the true volume of oxygen consumed.

RESULTS. When the work of the heart is increased by increasing the venous filling, we find invariably, in confirmation of the original work of Starling and Visscher, that the energy liberation as measured by the

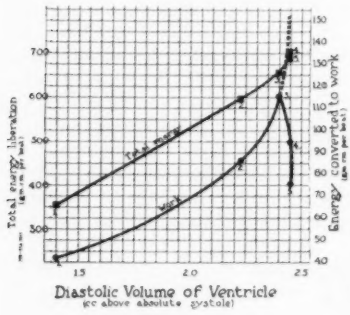

Fig. 2

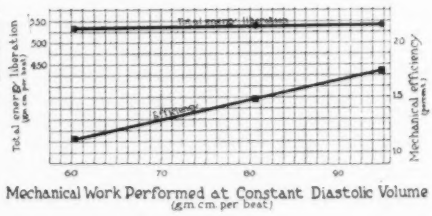

Fig. 3

Fig. 2. The relation between energy liberation and work and the diastolic volume. Experimental procedure in text.

Fig. 3. Work and energy liberation in relation to changes in arterial pressure at constant diastolic volume. The increasing efficiency occurred with a decrease in the pressure against which the ventricle was made to pump.

oxygen consumption varies with the work only when the work varies directly with the diastolic volume. In figure 2 are plotted the results of a typical experiment. One finds that when the contraction approaches isometricity, the apparent relation between work done and energy liberation vanishes. At the third point of observation the ventricle was working against a pressure of 55 cm. H_2O and was ejecting 2.07 cc. per beat. The arterial pressure was then raised to 60 cm. H_2O and the ejection fell to 1.43 cc., thus markedly diminishing the work accomplished. At the fifth observation the arterial pressure was raised still further, to 70 cm. H_2O, and again the work fell off. If the energy liberation were correlated with the work done, it should have decreased at least 20 per cent, instead of,

as it did, remaining exactly correlated with the diastolic fiber length. Or, conversely, if the work done were to be predicted from the total energy liberation, one would have expected at least 150 gm. cm. per beat instead of the falling off in work actually observed. Obviously there is a break in the apparent relation between work and energy liberation when the optimal arterial pressure is exceeded. There is, however, no break whatever in the continuity of the relation between initial fiber length and energy production. This experiment is entirely typical of seven such complete experiments upon fresh normal hearts. We have always found that as conditions approach those for isometric contraction, the work done falls off, but the energy liberation remains a function of the fiber length. The values for arterial pressure above which the work of the heart falls off, we have found to lie between 50 and 70 cm. H_2O for hearts in good condition. This result confirms Peserico (1928). After passing the "optimum" pressure, the ejection is limited and isometricity is approached. If the "Fenn effect" were to be observed in cardiac muscle, it should make itself apparent in a diminished oxygen consumption under those conditions. In figure 3 are plotted the results of an experiment in which the diastolic volume was kept constant and only the arterial pressure was altered between 50 and 60 cm. H_2O. As the pressure was raised the work accomplished fell off, while the total energy liberation remained constant within a very few per cent. In other words, under the crucial conditions for testing the influence of work done in contraction upon the energy liberated during the contraction, it appears that there is no connection.

Rigler (1932) has attempted to correlate the work of the heart with the systolic volume. Recalculating the data of Peserico (1928) he finds the work performed directly proportional to a fractional power of the systolic volume. Obviously this is a fortuitous relationship, since when the contraction approaches isometricity, as in our figure 2, observations 4 and 5, the work varies inversely with the systolic volume.

The fundamental problem as to the regulation of energy liberation in contraction in muscle is undoubtedly very much complicated by our uncertainty regarding the mechanism of transformation of chemical into mechanical energy. If we had a picture of the actual process occurring, we could much better postulate from known facts the rules regarding the rate of energy liberation. At the present time we are probably not justified in going further than to make observations upon phenomena which can be shown to be correlated. It seems unlikely, as Stella has pointed out, that there should be any fundamental difference between striated and cardiac muscle with respect to the mechanism of regulation of energy liberation. Still, since we must confine ourselves to the facts of the case in our present state of knowledge concerning the character of the muscle machine, it seems that, provisionally at least, we must conclude that there is either some superficial or fundamental difference between the

behavior of striated and cardiac muscle in this regard. It may not be amiss to point out that this may be exceedingly superficial and consist in such a difference as a failure of striated muscle to be completely excited in isometric contractions because of local pressure blocks to conduction, which would probably not occur in cardiac muscle, where the character of the loading is entirely different. It is very hard to rule out with certainty some of these factors in the experimental approach to the problem. It is therefore not desirable to over-emphasize the significance of this apparent difference which we have observed between striated and cardiac muscle. It seems important only to insist upon the fact itself of the dependence of the magnitude of energy liberation in cardiac muscle upon fiber length rather than work accomplished.

This interdependence of fiber length and energy liberation is particularly important in the applied physiology of the heart. It was pointed out by Starling and Visscher and is completely confirmed in these experiments upon the turtle's heart that when the physiological condition of the heart alters, so that the ventricle is able to perform less work at a given initial fiber length, the energy liberation is not lessened. The fundamental defect in the failing heart is, therefore, not an inability to liberate energy, but only a failure to utilize it in work. The energy supply is adequate but the machine for converting it into work is defective.

SUMMARY

1. An improved method has been used in measuring the oxygen consumption, work performance, and diastolic volume of the tortoise ventricle in order to test the influence of work done upon total energy liberation in cardiac muscle.

2. It is found that under crucial conditions the energy liberation is independent of work performed, but is entirely dependent upon fiber length during the chemical changes associated with energy liberation in contraction.

BIBLIOGRAPHY

CLARK, A. J. AND A. C. WHITE. 1928. Journ. Physiol., lxvi, 185.
EISMAYER, G. AND H. QUINCKE. 1930. Zeitschr. Biol., lxxxix, 513.
EVANS, C. L. 1914. Journ. Physiol., xlix, 378.
FENN, W. O. 1923. Journ. Physiol., lviii, 175.
HEMINGWAY, A. AND A. R. FEE. 1927. Journ. Physiol., lxiii, 299.
KNOWLTON, F. P. AND E. H. STARLING. 1912. Journ. Physiol., xliv, 206.
LÜSCHER, E. 1920. Zeitschr. f. Biol., lxxii, 107.
PESERICO, E. 1928. Journ. Physiol., lxv, 146.
RHODE, E. 1912. Arch. f. exp. Path. u. Pharm., lxviii, 401.
RIGLER, R. P. 1932. Pflüger's Arch., ccxxix, 282.
STARLING, E. H. 1915. Linacre Lecture on the law of the heart.
STARLING, E. H. AND M. B. VISSCHER. 1927. Journ. Physiol., lxii, 243.
STELLA, G. 1931. Journ. Physiol., lxxii, 247.

THE EFFECT OF EXPERIMENTAL MASSIVE HEMORRHAGES ON THE SIZE OF THE RED BLOOD CELL IN DOGS[1]

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During recent years much emphasis has been placed on the determination of the size of red blood cells in the various clinical forms of anemia; and most hematologists now agree that average cell diameter or mean corpuscular volume determinations constitute an important part of a complete blood study, probably just as important as the conventional red cell count and hemoglobin determination. Because of its importance in diagnosis and treatment, the average size of erythrocytes has even been made the basis of a classification of anemias which divides them into macrocytic, normocytic and microcytic types. There seems to be general agreement that pernicious anemia, the anemia of dibothriocephalus latus infestation and that of sprue are associated with a definite increase in the size of the erythrocyte, whether expressed in terms of average cell diameter or of mean corpuscular volume; and macrocytosis with achlorhydria has come to be regarded as almost sufficient for a diagnosis of pernicious anemia, while so-called secondary anemia is said to be associated with a decrease in cell volume or normal volume (1), though it is obvious that the many varying causes of secondary anemia might well provoke differences in the size response.

In the experimental anemias induced by hemorrhage, various conflicting reports indicate the difference of opinion that now exists regarding the effect of hemorrhage on the average size of the erythrocyte. This will be discussed in more detail later.

In the course of some unpublished experiments on hemopoiesis in which direct measurement of red cell diameters was included, a number of observations made us question the more commonly held view that red cells become smaller after experimental hemorrhage and in some of the so-called secondary anemias seen clinically. To illuminate this question, the following experiments were carried out.

METHOD. Two series of dogs, three in each series, were used as follows: After the normal blood picture had been sufficiently established, 50 per

[1] Read before the American Society of Experimental Pathology, Philadelphia, April 29, 1932.

cent of the determined blood volume was removed from the jugular vein of each of the two test dogs in the first series, the third dog being kept as a control throughout the entire experiment. The second series of three dogs included two test animals which were bled to a maximum; 60 per cent of the determined blood volume was removed from one of the test dogs on one day and an additional 30 per cent of the remainder was removed on the next. The second test dog in this group was bled 50 per cent of the blood volume on one day and an additional 30 per cent of the remainder on the next. The third dog was kept as a control.

Direct observations included the erythrocyte count, hemoglobin estimation (Sahli), reticulocyte count (brilliant cresyl blue, wet method), hematocrit readings (whole blood centrifuged for 10 minutes in van Allen tubes 2873 revolutions per minute),[2] plasma volume determinations (modified vital red method) (3) and direct measurement of unfixed red blood cell diameters. From these direct observations, total cell volume, total blood volume and mean corpuscular volume were calculated; the latter according to the formula of Wintrobe (2).

$$\text{Mean Corpuscular Volume in cubic micra} = \frac{\text{Volume of packed erythrocytes in cc. per liter of blood.}}{\text{Number of R.B.C. in millions per c.mm.}}$$

Erythrocyte diameters were measured in fresh thin wet smears, ringed with vaseline, using the ocular filar micrometer. One hundred cells were usually measured in each observation, and observations made at frequent intervals before bleeding to establish the normal for the animal, daily for about ten days after bleeding and at irregular intervals thereafter up to the end of the observation period. In spite of the tedious nature of this test, a total of over thirteen thousand cells was measured in this way. Being particularly interested in the effect of massive hemorrhages on the size of the red blood corpuscles, we included the other blood studies, not so much to add to the numerous similar observations previously reported but as a background for the interpretation of changes in size of red blood cells in the light of other changes in the blood picture. Changes in plasma pH, osmotic pressure, etc., which might temporarily influence the size of the cell, could not be studied. We have two series of observations, then, which indicate the size of the erythrocytes, a direct measurement of diameters and an indirect calculation of mean corpuscular volume.

Results. Preoperative (i.e. "normal") determinations on the 6 dogs, gave an average diameter of 7.8μ (extremes 6.6 to 8.9μ) and an average corpuscular volume of 58 cu. μ. In the first series, in which the hemorrhage amounted to 50 per cent of the blood volume, the table of weekly

[2] It had previously been ascertained that in no case was the result changed by longer centrifugation.

TABLE 1

*Effect of massive hemorrhages on the blood picture of the dog. Series I (weekly averages)**

WEEK ENDING	RED BLOOD CELLS (MILLIONS)	HEMOGLOBIN (PER CENT SAHLI)	RETICULOCYTE (PER CENT)	HEMATOCRIT (PER CENT)	AVER. RED BLOOD CELL DIAMETER (MICRA)	PLASMA VOLUME (CC.)	CELL VOLUME (CC.)	BLOOD VOLUME (CC.)	MEAN RED BLOOD CELL VOLUME (CU. μ)	BODY WEIGHT (KILO)	BLOOD VOLUME (PER CENT BODY WEIGHT)
Dog 469											
1- 9-32	6.40	100	0.1		7.7	579	521	1,100		13.2	8.3
2- 8-32	7.30	100	0.2	39	7.8	555	505	1,061	53	12.4	8.0
2- 9-32	Bled 540 cc. approximately one-half total blood volume										
2-17-32	4.00	57	1.3	24	7.8				62	13.0	
2-24-32	4.51	68	1.4	33	7.8	688	328	1,016	72	12.5	8.0
3- 1-32	5.14	71	0.6	38	8.0	513	431	945	72	12.9	
3- 8-32	5.74	83	0.2	43	7.9				75		
3-15-32	6.67	93	0.1	43	7.8				65		
3-22-32	6.79	98	0.2	48					70		
3-29-32	6.90	103	0.1	50	7.7				71		
4- 9-32						561	510	1,072		12.6	
Dog 603											
1- 9-32	7.18	93	0.2		7.7	665	599	1,264		14.8	8.5
2- 8-32	6.39	87	0.1	37	7.7	620	501	1,122	59	13.4	8.6
2- 9-32	Bled 625 cc. approximately one-half total blood volume										
2-17-32	4.05	55	1.3	26	7.8	762	282	1,045	63	14.5	7.2
2-24-32	4.61	62	0.7	32	7.8	730	337	1,067	69	13.6	7.8
3- 1-32	4.79	63	0.2	34	8.0	710	343	1,054	64	13.9	7.6
3- 8-32	4.84	65	0.4	33	7.9				69		
3-15-32	5.10	72	0.1	36	7.9				73		
3-22-32	5.60	83	0.1	42					76		
3-29-32	6.00	85	0.1	42	7.8				67		
4- 9-32						628	503	1,131		14.2	
Dog 602—control											
1- 9-32	7.40	104	0.2	44	7.8	476	418	889	59	13.2	6.7
2- 8-32	7.41	101	0.2	45	7.7	505	531	1,036	60	12.5	8.3
2- 9-32	Control dog										
2-17-32	7.38	101	0.2	40	7.7	526	601	1,127	56	12.6	8.9
2-24-32	7.21	94	0.2	40	7.8	Hemolysis			56	12.7	
3- 1-32	7.08	90	0.1	38	7.9	571	449	1,021	54	12.1	8.4
3- 8-32	7.01	85	0.2	40	7.8				57		
3-15-32	6.86	92	0.2	41	7.9				60		
3-22-32	6.95	95	0.1	44					64		
3-29-32	6.95	94	0.2	44	8.0				65		
4- 9-32						526	584	1,110		12.5	

*As there were 45 sets of observations on each animal in this series, they have been condensed to weekly averages for the sake of brevity, inasmuch as no significant change was obscured thereby.

TABLE 2

Effect of massive hemorrhages on the blood picture of the dog. Series 2

DATE (1932)	DOG 930								DOG 682								DOG 681 (CONTROL)							
	Red blood cells (millions per cmm.)	Hemoglobin (per cent Sahli)	Reticulocyte (per cent)	Hematocrit (per cent)	Average red blood cells diameter (micra)	Diameter extremes		Mean red blood cells volume (cu. micra)	Red blood cells (millions per cmm.)	Hemoglobin (per cent Sahli)	Reticulocyte (per cent)	Hematocrit (per cent)	Average red blood cells diameter (micra)	Diameter extremes		Mean red blood cells volume (cu. micra)	Red blood cells (millions per cmm.)	Hemoglobin (per cent Sahli)	Reticulocyte (per cent)	Hematocrit (per cent)	Average red blood cells diameter (micra)	Diameter extremes		Mean red blood cells volume (cu. micra)
						Minimum	Maximum							Minimum	Maximum							Minimum	Maximum	
3–17	8.33	106	0.4						7.79	95	1.3		7.8				7.98	101	0.8					
3–18	8.00	103	0.1	49	7.6	6.6	8.5	61	7.67	100	1.1	44	7.8	6.5	9.1	58	8.22	102	1.0		7.7	6.5	9.4	
3–19	7.85	103							7.61	94							7.88	100						
3–22	7.76	100	0.3	45	7.6	6.5	8.6	59	7.59	96	1.8	45	7.7	6.7	9.3	60	7.91	102	1.1	44	7.7	6.8	8.7	56
3–24	7.78	102	0.1						7.50	95	1.6	44				59	7.80	95	0.9	44				56
3–25	7.98	103	0.1		7.5	6.5	8.5		7.75	102	1.9		7.7	6.5	9.0		7.72	95	1.5		7.6	6.9	8.5	
3–31	7.85	98	0.1	44	7.6	6.7	9.1	57	7.72	100	1.4	47				61	7.74	98	2.1		7.6	6.7	9.0	
4–1	7.73	95	0.1	40	7.5	6.1	8.4	52	7.59	98	0.7	43	7.9	6.5	9.1	57	7.48	92	3.0	41	7.7	6.9	8.7	55
4–4	770 cc. blood removed				7.6	6.5	8.8		1,100 cc. blood removed												7.7	6.3	9.1	
4–5	5.17	74	1.2	31				60	5.44	75	1.6	32				59	7.54	94	1.8	42				
4–5	505 cc. blood removed								535 cc. blood removed															
4–6	3.46	52	8.3	24	7.9	6.6	9.9	70	3.63	48	1.3	27	8.0	6.8	9.4	74	7.17	87	1.5	43	7.6	6.3	8.8	56
4–7	3.65	48	5.4	22	8.1	6.4	10.8	61	3.47	42	1.6	21	8.2	7.0	9.5	61	6.99	84	1.1	42	7.7	6.7	8.6	
4–8	3.46	46	6.4	21	8.2	6.5	11.3	61	3.32	42	3.4	22				66	6.66	80	1.5	40	7.9	7.1	8.8	60
4–9	3.29	45	7.6	20	8.2	6.6	11.0	63	3.10	40	7.5	21	8.0	6.8	10.5	70	6.91	85	0.7	36	7.8	7.0	8.9	61
4–11	3.48	48	9.8	22	8.2	6.6	10.8	64	3.37	44	5.8	26	8.2	7.1	9.8	77	6.80	80	1.1	31	7.8	6.5	9.0	61
4–12	3.52	48	7.5	29	8.3	7.1	10.4	84	3.44	47	8.6	30	8.2	6.9	9.9	88	6.67	75	1.5	32	7.9	6.6	9.4	52
4–13	3.71	52	4.0	28	8.2	6.7	10.9	76	3.78	56	7.7	30	8.2	7.0	10.4	80	6.79	76	0.9	35	7.9	6.7	9.2	45
4–14	3.78	55	5.4	33	8.3	6.1	9.6	87	4.03	60	5.4	33	8.3	6.9	10.3	83	6.84	78	0.4	36	7.9	6.5	9.2	48
4–15	3.79	52	4.9	31	8.1	7.0	10.2	82	4.28	62	6.4	31	8.0	6.5	10.0	66	6.94	82	1.3	34	7.7	6.6	8.3	52

4–18	4.08	54	4.3	30	8.1	6.9	10.0	74	4.72	67	1.6	33	8.0	6.5	9.5	71	6.90	79	1.8	37	7.8	6.9	9.2	53
4–20	4.19	58	4.2	31	8.0	6.6	9.4	75	4.90	72	3.0	38	7.9	7.0	8.9	79	6.82	83	3.3	39	7.9	6.8	9.2	58
4–22	4.74	67	6.1	30	8.0	6.3	9.2	64	5.26	74	2.4	36	7.9	6.7	9.4	69	6.61	79	6.0	34	7.9	6.9	9.2	52
4–23					8.0	6.8	9.2						7.8	6.8	8.8						7.9	6.7	9.2	
4–25	5.07	75	4.0	39	7.9	6.7	9.3	77	5.79	85	2.3	42	7.9	6.5	9.4	72	6.23	74	1.9	35	7.8	6.5	9.2	56
4–27	5.20	80	3.9	38				73	6.29	88	1.1	43				68	6.01	66	1.5	31				51
5–2	5.87	87	3.5	40	7.9	6.4	8.9	69	6.96	94	0.6	45	7.9	6.7	8.8	64	5.85	67	3.2	31	7.9	6.8	9.6	54
5–4	6.57	91	0.5	40				62	7.01	95	0.2	42				59	5.60	62	3.0	32				57
5–6	6.65	91	0.7	40	7.9	6.7	9.2	61	7.24	94	0.2	42	7.8	6.7	8.8	58	5.03	60	4.9	31	7.8	6.5	8.6	62
5–10	6.76	90	0.7	43				64	7.63	94	0.1	50				66	4.72	57	2.9	28				60
5–13	7.11	94	0.7	44	7.8	6.6	9.5	63	7.77	95	0.1	46	7.8	6.9	8.7	60	4.99	65	3.9	29	7.8	6.8	8.8	69
5–17	7.61	100	0.1	46				61	7.79	98	0.7	48				62	4.29	58	3.6	29				68
5–20	7.78	98	0.1	43				55	8.17	100	1.0	46				56	4.09	54	4.0	27				67
5–25	7.70	100	0.1	43				56	7.95	99	0.6	46				58	4.29	53	2.2	27				65
5–27	7.61	95	0.05	46	7.7	6.8	9.0	61	8.03	95	0.2	43	7.6	6.9	8.7	54	4.60	52	1.6	25	7.6	6.9	8.6	55

Plasma volume	853 cc.	Plasma volume	900 cc.	Plasma volume	878 cc.
Total cell volume	741 cc.	Total cell volume	936 cc.	Total cell volume	706 cc.
Blood volume	1,595 cc.	Blood volume	1,836 cc.	Blood volume	1,583 cc.
Body weight	18 K.	Body weight	19 K.	Body weight	17 K.

averages (table 1) shows the expected fall in erythrocyte count and hemoglobin, a noteworthy reticulocyte rise, a fall in total cell volume, an increase in plasma volume and a more or less constant total blood volume. It took about eight weeks for the blood picture to reëstablish itself after the hemorrhage.

EFFECT OF HEMORRHAGE ON "SCATTER" OF DIAMETERS OF ERYTHROCYTES.

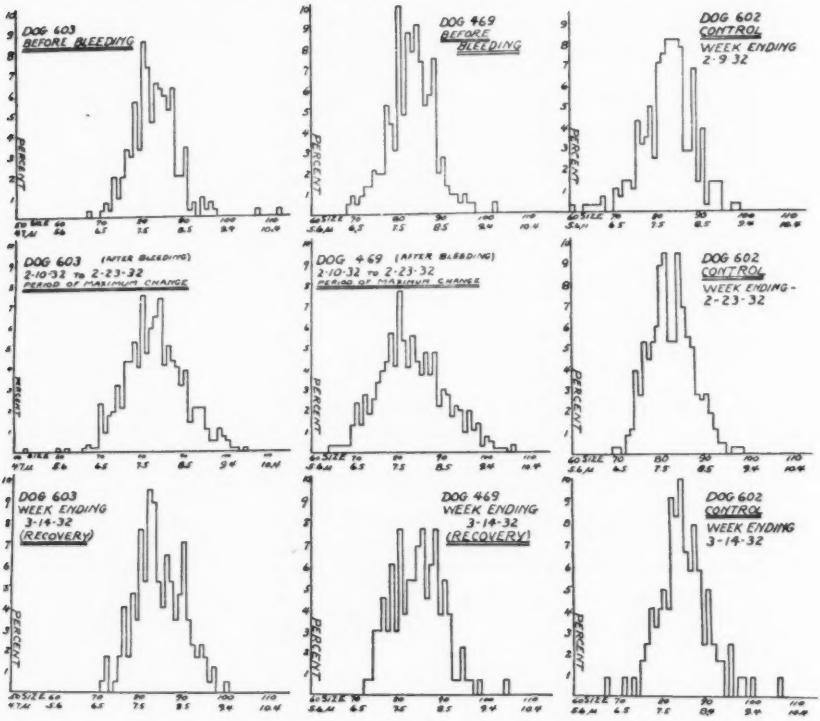

Fig. 1

The average diameter of the red blood cells was but doubtfully increased by this blood loss. Nevertheless a striking change was apparent with one glance at the smears during the immediate post-bleeding period when there were many small cells and about as many large ones which kept the average cell diameter almost the same. This marked anisocytosis can probably be best expressed graphically in the modified Price-Jones percentage distribution curves (figure 1) (3), designed to show the "scatter" of erythrocyte diameters. It was during the period between the second and thir-

teenth day after the hemorrhage that the most striking change in the size of the red blood cells was noted. The calculated mean corpuscular volumes, on the other hand, showed a more distinct increase in the size of the red blood cells than did the diameter measurement, thus tending to confirm the view that this is a more sensitive indicator of change in size

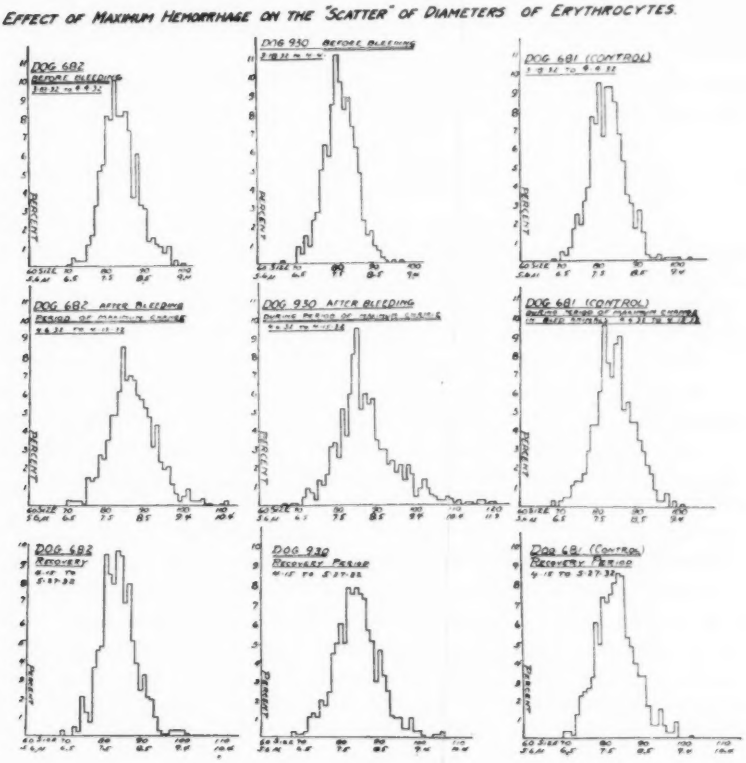

Fig. 2

in spite of the extra possibility of error in the two observations required in making the corpuscular volume calculation.

In the second series in which two dogs were bled to the limit of safety, there followed a definite increase in average cell diameter (table 2) as well as a striking increase in scatter (figure 2). This time the large cells seemed to outnumber the small ones. An analysis of the data shows that the maximum change occurred during the nine days immediately following the hemorrhage. During this period there was also an increase in the mean

corpuscular volume. The red count, hemoglobin, reticulocyte count and hematocrit readings underwent the changes one naturally expects after severe hemorrhage.

Discussion. Our observations indicate that anisocytosis or *increase in "scatter"* of erythrocyte diameters is the most striking change in the morphology of the red blood cell after sudden massive hemorrhage, and that with extreme loss of blood, there is a definite *increase* in average cell diameter and volume.

In contrast to the marked changes in the size of red cells seen in some of the longer continued clinical anemias, of which pernicious anemia is the classical example (4), we are dealing here with slighter changes and it is not at all unlikely that some of the finer variations are lost in the probable error of the method. The results obtained in the pre-bleeding observations and in the two controls convince us, however, that the changes in the test animals were real and significant of the response to this kind of blood loss.

With marked variation in the size of red cells after hemorrhage, it would of course be easy to get extremely wide variations in average cell diameters from a single smear if one, perhaps unconsciously, concentrated on the smaller or the larger cells. We have attempted to overcome this difficulty by measuring a true continuous series of cells as they appeared in the field. It is obvious, too, that when there are many small and large cells in the smear, checks on the same blood by different observers are not apt to be so good as when the cells are nearly all of the same size. This error varies inversely with the number of cells measured.

The indirect method of expressing size of red blood cells in terms of mean corpuscular volume furnishes a good check on the laborious direct measurement of diameters and is probably adequate for clinical purposes. It has the advantage of simplicity and the disadvantages of not demonstrating "scatter," and of being an indirect record based upon two observations where the errors may be cumulative. It is probable, too, that some of the simpler methods of determining cell diameter would be adequate for clinical purposes, allowance being made for the shrinkage of fixed cells, but we have no direct evidence on these points.

The usual precautions in standardizing technique as far as possible—which we have found to be of prime importance in obtaining trustworthy results—have been scrupulously carried out. Nevertheless, we recognize that both technical and biological variables that have not been entirely controlled have contributed to the discrepancies in the results. Like much of the work in this field, it should be considered as semi-quantitative in nature and evaluated accordingly. The various precautions—standardized apparatus, conditions of obtaining samples, same individual doing the test at the same time of day under identical conditions, minimizing

the dog's emotional strain, etc.—will not be further detailed here, though their great importance should be emphasized.

No attempt will be made at this time to analyze in detail the extensive literature on this subject. Indeed the conclusions to be deduced from such a survey would be of doubtful value, since many different methods (5), (6) have been used to measure the size of the cells, the extent of the hemorrhage varies with the investigator and various secondary anemias of different etiology and obscure pathogenesis have been lumped together. Even in the study of erythrocyte size in hemorrhagic anemia, the amount of blood lost is in some studies entirely and often necessarily unknown.

Price-Jones, who was one of the first to emphasize the clinical importance of measurement of red blood cell diameters, contributed much of the early work in this field. For instance, in one of his early experiments (7), he bled eight rabbits of 54 to 76 per cent of the oxygen capacity over a period of three days and found the hemoglobin average of 80 per cent (human standard) reduced to an average of 30 per cent, with the lowest reading on the day after the last hemorrhage. In this experiment, the average diameter of one hundred cells showed no change during the period of bleeding; but on the day after the last bleeding, the diameter began to increase from a pre-bleeding average of 6.2 micra up to 7.6 micra on the eighth day. In some later work, however (3), in which he studied the average diameter of red blood cells in ten cases of human anemia following hemorrhage of unknown amounts from various sources (gastric and uterine), the average cell diameter was found to be smaller than in the twenty healthy controls similarly studied.

In a series of twenty-seven dogs rendered anemic by bleeding (amounts not given), Mayerson and Laurens (8) found a decrease in corpuscular volume. In their study of six rabbits with hemorrhagic anemia, on the other hand, Ponder and Miller (9) report a small but significant increase in the size of the red blood cells. Like ours the blood cells were examined in plasma; measurements were made from photographs of known magnification. Leichsenring and Honig (10) made seven dogs anemic by the removal of approximately half of their calculated blood volume and found the diameter of the red cells (studied in a counting chamber, mixed with Hayem's fluid) to be decreased during the regeneration period from an average of 6.9 micra before bleeding to 6.1 micra at the end of the post-bleeding period. Many other examples could be cited from the literature to emphasize the difference of opinion on this subject which, as mentioned above, becomes especially important when the size of the red blood cell is being made the basis for a classification of the anemias. The true story of the effect of hemorrhage on the size of the red cell will doubtless shortly be forthcoming. In the meantime, it is impossible to say how much the apparent discrepancy of results should be laid to incorrect or incomplete

observation and how much to the fact that different amounts of hemorrhage spread over different periods of time may produce different responses, in terms of size, on the part of the erythropoietic factory. It is easily conceivable, for instance, that a diseased bone marrow, or one undernourished as the result of any one of various long continued secondary anemias, might put out smaller erythrocytes than a healthy marrow suddenly subjected to an extreme demand. Here, too, the whole unexplored field of relative availability of material for stroma or for hemoglobin formation in the body might easily prove the decisive factor in the determination of size of the erythrocyte. From such points of view, even the same experimental procedure might conceivably produce opposite effects in different individuals or in stages of the process in one individual if factors contributing to hemopoiesis were sufficiently changed.

SUMMARY

The effect of massive hemorrhages on the blood picture of dogs was studied with special reference to the size of the erythrocyte. In one group, dogs bled half their determined blood volume showed a marked increase in scatter of erythrocyte diameters and only a slight increase in average cell diameter, as determined by direct measurement with the ocular filar micrometer. The calculated mean corpuscular volume was found to be slightly but definitely increased. The two test animals in the second group which were bled to the limit of safety showed a definite increase in average cell diameter as well as an increase in "scatter" and volume.

In view of the complex and varied nature of human secondary anemias, it is unwise and probably incorrect to group them as "microcytic" in type. The proposed classification of the anemias into "macrocytic," "normocytic" and "microcytic" is regarded as premature.

BIBLIOGRAPHY

(1) Grosh, L. C. and J. L. Stifel. Arch. Int. Med., 1925, xxxvi, 874.
(2) Wintrobe, M. M. Amer. Journ. Clin. Path., 1931, i, 147.
(3) Price-Jones, C. Journ. Path. and Bact., 1922, xxv, 487.
(4) Price-Jones, C. Journ. Path. and Bact., 1929, xxxii, 479.
(5) Ponder, E. and G. Saslow. Journ. Physiol., 1930, lxx, 18.
(6) Haden, R. L. Journ. Lab. Clin. Med., 1931, xvi, 976.
(7) Price-Jones, C. Journ. Path. and Bact., 1911, xvi, 48.
(8) Mayerson, H. S. and H. Laurens. Journ. Nutr., 1931, iii, 453.
(9) Ponder, E. and W. G. Millar. Quart. Journ. Exp. Physiol., 1928, xix, 145.
(10) Leichsenring, J. M. and H. H. Honig. This Journal, 1931, xcviii, 636.

RHYTHMIC ARTERIAL EXPANSION AS A FACTOR IN THE CONTROL OF HEART RATE

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ARGUMENT AND PREVIOUS WORK. The fact has been satisfactorily established that an increase in arterial pressure sets up reflexes from certain regions of the arterial system which cause a slowing of the heart or caliber changes in the peripheral arterioles. But, until recently, the mechanisms through which the nerve terminals in the arterial walls are excited have received less thought and attention. It is the current impression that stretching of the elastic arterial walls supplies some form of stimulus to the sensory nerve terminals ending within them. A little reflection makes it clear, however, that such a statement falls far short of offering a complete physiological concept of nerve end stimulation.

In the first place, the arterial walls are never in a constant state of distention because a mean or constant arterial pressure does not actually exist. Consequently, an increase of pressure does not imply that the arterial distention is shifted from one constant state to another. On the contrary, at any so-called mean arterial pressure level the elastic walls are periodically stretched and relaxed to variable degrees; in fact, the magnitude of this cyclic change may be greater than the measured increase in mean pressure required to produce reflex changes in heart rate. It follows, either that an average change in the state of arterial distention acts as a constant mechanical stimulus or that the frequency, magnitude and steepness of the systolic and diastolic variations in calibre are concerned. The former is the more commonly accredited agent, but it has less experimental evidence in its favor than the latter. Indeed, if a more or less constant degree of tension on intra-arterial nerve terminals is an effective means of eliciting a constant stream of afferent impulses, then their mode of response and adaptation apparently differs essentially from that of other sensory nerve endings.

Such reflections, together with Adrian's demonstration (1926) that the depressor nerve discharges two groups of impulses during each cardiac cycle, suggested a study of the relative importance of mean pressure and pulse pressure in the reflex control of heart rate and blood pressure in 1930.

Unfortunately a good part of the experimental work preceded any knowledge of the beautiful results of Bronk and his associates on the character of arterial end-organ discharges. Hence, the performance of experiments could not be guided by these discoveries. With the successive publication of these results a report of these investigations was purposely deferred in order that an interpretation might be guided by and harmonized with them.

Briefly reviewed, Bronk and Kaltreider (1931) first showed that the depressor nerve discharges two series of impulses coincident with the rise of pressure and the incisura respectively, no activity persisting during diastole. When, however, arterial pressure was increased considerably by epinephrin the number of discharges increased and the second group continued during diastole. More recently, Bronk and Stella (1932–1932a) recorded similar discharges from the sinus caroticus nerve both in the nerve as a whole and in single fibers. They concluded that the degree of activity is influenced both by variations of pressure during each cardiac cycle as well as by the mean pressure; but if we may venture to interpret their experimental findings, the evidence chiefly supports the conclusion that the mean stretching of arteries is the main factor. When the mean pressure is raised considerably by epinephrin the afferent impulses become quantally greater both because the duration of discharge in individual fibers increases and because a larger number of end organs apparently come into play. Any doubt that these effects may be due to chemical effects of drugs employed rather than pressure changes is removed by the fact that similar results were obtained by perfusing the carotid sinus at different constant mean pressures. Furthermore, the fact was clearly demonstrated that intra-arterial end-organs adapt very slowly, the discharge continuing indefinitely at an only slightly decreased frequency.

Despite this progress, several gaps still remain in our understanding of the ultimate mechanisms that reflexly moderate heart rate and blood pressure: 1. The rôle that alterations of pulse pressure play when mean pressure remains unaltered or changes in a reverse direction has not been studied; yet such dynamic conditions are probably of more frequent occurrence in normal life than the extreme changes of mean pressure so generally investigated. 2. While it can be surmised that an increase in total number of afferent impulses per minute impinging on the bulbar centers produces somewhat proportional reflex effects on the heart and blood vessels, the fact has not actually been demonstrated. Since our experiments contain information on these points and moreover elucidated pertinent dynamic questions, their publication is justified on other grounds than that of being merely confirmatory or contradictory to observations of others.

I. THE RÔLE OF PULSE VARIATIONS IN THE "WHOLE ANIMAL". It was necessary in the first place to establish whether changes in pulse pressure

without modification of mean pressure affect the heart rate in the "whole animal" for unless this proved to be the case no necessity existed for pursuing the investigation further. In the first series of experiments an effort was therefore made to study the changes in heart rate which are reflexly or otherwise initiated when mean pressure and pulse pressure were made to vary as independently as possible through a variety of experimental procedures.

Methods. Numerous tests were made on 15 different dogs. They were anesthetized with morphine and sodium barbital, the latter anesthetic being used, since visceral reflexes appear to be better maintained than with other permanent anesthetics (Pearcy and Weaver, 1927). After institution of artificial respiration, the chest was opened and a calibrated optical manometer was inserted into the aorta through the left subclavian artery. The customary precautions in the use of such apparatus, as outlined by Wiggers (1928), were adopted. Variations in systolic and diastolic pressures were produced by mechanical compression of the thoracic aorta, by saline infusion, and by compression of the inferior vena cava. In addition, pressure changes were produced by creation of temporary aortic insufficiency by methods already described by Wiggers and his associates (1915, 1930). Naturally, great care was taken to leave all nerves intact and to maintain the circulation in good condition. While pressure curves were being recorded, respiration was temporarily discontinued, so as not to complicate the pressure curves by respiratory disturbances.

In studying the effects of changes in mean pressure and pulse pressure, alterations in heart rate were used as criteria. This was determined by careful measurement of a series of pulse beats only one of which is reproduced in each of the various illustrations.

In analyzing the optical pressure curves, the systolic and diastolic pressures and their differences, the pulse pressure could be directly compared or translated into millimeter of mercury through application of a calibration scale. In order to interrelate changes in pulse pressure thus established with variations in mean pressure it was necessary to adopt a rather precise conception of the mean pressure upon which the mean distention of the arterial wall depends. Mathematically, it is the sum of an infinite series of successive pressures, existing during the cardiac cycle, divided by an infinite number of assumed pressure variations. This implies that the actual mean pressure is determined not only by the numerical systolic and diastolic pressures but by the form of the pressure curve as well. In accordance with this concept mean pressure was calculated by measuring the area beneath the curves to a zero base line by means of a planimeter and dividing by the horizontal distance. This gave the mean pressure height in millimeters which, by use of a calibration scale, is easily

converted into millimeters of mercury. The lines drawn in figures 1, 2 and 3 indicate the areas measured.

Results. Obviously, only those experiments in which mean pressure remained relatively constant or changed in a direction the reverse of pulse pressures are crucial in nature. It must be understood however that only with great difficulty can one alter pulse pressure experimentally without inducing simultaneous changes in mean blood pressure. Hence, it was necessary to discard many data because it was impossible to decide whether mean pressure or pulse pressure changes chiefly determined heart rate changes. Thus, compression of the vena cava or of the aorta resulted in an altered pulse pressure but the results of such experiments could not be evaluated because the mean pressure varied in the same direction as

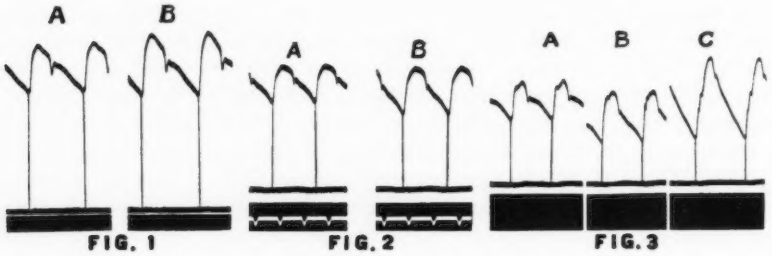

Fig. 1. Experiment 5; 1AB. Optical pressure curves from aorta illustrating the effect of increased pulse pressure during saline infusion on heart rate. A, control. B, infusion (causing slight rise in mean blood pressure). Time 0.01 second.

Fig. 2. Experiment 14; 2CD. Same as figure 1. A, control. B, infusion resulting in fall of mean blood pressure. Time 0.2 second.

Fig. 3. Experiment 25; 3BC. Optical pressure curves from aorta showing: A, control. B, effect of experimental aortic insufficiency. C, same as B after compensatory aortic compression. Time 0.2 second.

pulse pressure, hence the changes in heart rate induced might be due to these two factors which could not be separated. The observations obtained were useful, however, in establishing the minimum changes in mean pressure which just suffice to produce a recognizable variation in heart rate, say five beats per minute. It was found that in no case were rate changes noted when the extent of the mean pressure rise was 4 mm. of mercury or less. It was usually necessary to lower the mean pressure slightly more to induce acceleration, namely, from 4.5 to 5 mm. of mercury. These mean pressure changes are somewhat greater than those in the sinus caroticus which Heymans and Bouckaert (1930) found to cause reflex alterations of the peripheral vascular tonus. Changes in mean pressure of 10 mm. Hg in either direction in animals with good reflex response always

caused a change of rate of 5 to 12 beats per minute, unless offset by contrary changes of pulse pressure.

Effect of saline infusion. Since past experience had demonstrated that an infusion of isotonic saline solution at body temperature may be given at such a rate that mean pressure recorded with a mercury manometer remains practically unaltered while pulse pressure is increased, the effects of such infusions were studied. An analysis of 25 experiments showed, however, that when actual mean pressures were carefully calculated, such a condition was fully realized in three experiments only. But, in these, the pulse pressure increased only 5,5 and 10 mm. Hg. Cardiac slowing to the extent of 8 beats per minute occurred only in the last, the others showed no change in rate. These rather negative results might of course be due to the rather small increase in pulse pressure. In four other experiments both mean and pulse pressure increased so much that the causative factor in the resultant cardiac slowing could not be determined.

In 10 experiments the mean pressure rose only slightly, i.e., 2 to 7 mm. Hg and the pulse pressure increased 7 to 12 mm. Hg. In 8 of these, a diminution of rate of 5 to 11 beats per minute was noted. An example is reproduced in figure 1. The control record (segment A) shows a systolic pressure of 121 mm., a diastolic pressure of 90 mm., a mean pressure of 108 mm. and pulse pressure of 36 mm. Hg. Heart rate is 158 per minute. During saline infusion (segment B) systolic pressure increased to 134 mm., diastolic fell to 86 mm., mean increased by 3 mm., and pulse pressure by 12 mm. Hg. The heart rate decreased 7 beats per minute. These effects may be contrasted with three other experiments in which mean pressure rose 11 to 33 mm. and pulse pressure increased 5 mm. of mercury or less. In these no change in heart rate occurred.

Any suggestion as to the dominant influence of pulse pressure that might be read into such results is somewhat counterbalanced by other observations. Comparison of two experiments in which pulse pressure increased by 4 and 23 mm. and mean pressures rose 5.5 and 3.6 mm. Hg respectively, showed corresponding decrease in heart rate of 8 beats per minute. In 3 cases in which a 2 to 11 mm. rise of mean pressure and an increase of 7 to 9 mm. Hg in pulse pressure occurred, the heart actually accelerated from 3 to 13 beats.

Three experiments, however, appeared to have greater significance. In these, mean pressures fell 17, 12 and 7 mm. Hg with corresponding increases in pulse pressure of 16, 4 and 11 mm. Hg respectively. Decreases in heart rates of 11, 8 and 10 beats per minute were found. The record of figure 2, the last of the above series, is typical of the average result in this group.

While such results were suggestive, it was recognized relatively early in the work that they are far from demonstrating any relationship between

rhythmic vascular expansion and reflex slowing of the heart and this mode of experimentation could not supply conclusive evidence on the thesis presented.

Effects of aortic insufficiency. The production of a temporary aortic insufficiency offers a simple method by which the effects of pulse pressure and mean pressure can be dissociated, and the effects on heart rate can be repeatedly determined on the same animal. In all cases in which no attempt was made to compensate artificially for the lesion, both mean and diastolic pressures fell and pulse pressures increased. Systolic pressure however was variable.

In 16 such experiments in which mean pressure decreased by 2 to 17 mm. Hg and pulse pressure increased between 9 and 47 mm. Hg, cardiac slowing of 5 to 12 beats occurred in 11 or 69 per cent of cases. Four were without change, and one showed an acceleration of five beats per minute. In these systolic pressure was slightly elevated. In fourteen experiments, in which mean pressure decreased 2 to 16 mm. Hg and pulse pressure increased from 11 to 32 mm. Hg, the systolic pressure remained unaltered. A decreased heart rate of 5 to 11 beats per minute occurred in 7 instances or 50 per cent. In 6 experiments there was no change in rate, while in one experiment the heart accelerated 5 beats per minute. One could interpret the latter case as one in which an imperfect neutralization of two antagonistic factors occurred; namely, an increased pulse pressure of 32 mm. Hg accompanied by a lowered mean pressure amounting to 22 mm. Hg.

Mean, systolic and diastolic pressures were all diminished in 9 experiments (while pulse pressure increased). Of these, the rate was decreased 5 beats each, in two (22 per cent) and remained unchanged in 7 cases.

The fact that a fall of mean pressure, sufficient when accompanied by decreased pulse pressure to cause cardiac acceleration (e.g., vena cava compression) causes no alteration in heart rate or even a definite slowing when pulse pressures are increased by an experimental aortic leak, suggests that pulse pressure is indeed an important, though probably not the only factor, which determines the changes in heart rate.

In many experiments an attempt was made to compensate for the fall of mean pressure during aortic insufficiency by compressing the thoracic aorta just above the diaphragm. An exact restoration of mean pressure to that existing before insufficiency is obviously only a matter of chance. Measurement of many records in which such attempts at restoring mean pressure were made showed that this was successfully accomplished in three instances only (cf. table 1). One of them (expt. 25–3BC) is shown in figure 3. Comparing segments A and B, the latter after production of an aortic leak, we note a marked decrease of systolic and diastolic pressures. The mean pressure decreased 23 mm. Hg (calculated)

and the pulse pressure increased 14 mm. (calculated). The heart rate remained unaltered at 231 beats per minute. In segment C, taken after compensatory aortic compression, measurement shows that the mean pressure is restored to that of segment A, systolic pressure is 28 mm. Hg higher, diastolic pressure 23 mm. Hg lower and pulse pressure 51 mm. Hg greater than in segment A. Calculations show that the heart rate decreased 44 beats per minute.

In two other experiments on the same animal (expts. 25–4DE and 2,G,H, table 1) similar results were obtained. In each, reduction of mean pressure combined with increase in pulse pressure failed to modify the heart rate, but when mean pressure had been restored to the control level and pulse pressures had greatly increased the heart rate decreased

TABLE 1

Summary: Aortic insufficiency + aortic compression

EXPERIMENT NUMBER	HEART RATE CHANGE	PRESSURES IN MILLIMETERS OF MERCURY			
		Systolic	Diastolic	Pulse pressure	Mean
25: 3BC	−44	+28	−24	+51	0
25: 4DE	−28	+30	−26	+56	0
25: 2GH	−24	+29	−29	+58	0
25: 1BC	−12	+34	−28	+62	−4
12: 7DE	−21	+16	−8	+25	+2
12: 6CD	−20	+14	−10	+24	+2
12: 6EF	−10	+6	−14	+20	−4
13: 6EF	−12	+12	−26	+38	−12
13: 9BC	−9	+8	−17	+25	−8
25: 2DE	−6	+9	−10	+19	−3
8: 6BC	0	+5	−14	+19	−3

significantly. The data of the remaining experiments recorded in table 1 show similar effects, though the mean pressure was only approximately equal to normal; indeed, there was only one experiment (expt. 8: 6,B,C) in which the reaction failed to occur.

Such results certainly demonstrate that a great intensification of pulse pressure without significant change of mean pressure is capable of slowing the heart.

Summarizing this portion of the investigation: experiments of different sorts indicate that, in a general way, an inverse relationship exists between mean pressure and heart rate as expressed by Marey's Law. Under normal conditions however agencies which increase mean pressure (increased discharge and increased peripheral resistance) likewise augment pulse pressures in the dog. On the other hand, those that lower mean pressure may either decrease pulse pressure (decreased output) or increase it

(moderate vasodilatation). Only in the latter case can the separate effects be studied. The production of a temporary experimental aortic leak however offers a simple method by means of which the effects of mean pressure and pulse pressure can be repeatedly tested in the same animal, for mean pressure declines while pulse pressure increases. The effects obtained varied from an acceleration or no change to a retardation, but the latter two effects were most common in experiments of this series. When, in addition, the mean pressure was restored approximately and in a few cases exactly, to normal levels by aortic compression, pronounced slowing of the heart followed in all tests except one. All these results demonstrate that alterations of pulse pressure *per se* can affect the heart rate. But while the demonstration of such an added control of heart rate in the "whole animal" is important in itself the experiments obviously allow no conclusions as to whether pulse pressure produces these effects by direct or reflex action, and if the latter, which of the many reflex arcs are concerned.

II. THE EFFECT OF PULSE PRESSURE VARIATIONS IN THE PERFUSE CAROTID ARTERY. The use of the "whole animal" did not offer promise in settling the question whether or not the cardiac slowing accompanying alterations of pulse pressure can be due to reflex action. Not only are dynamic changes which might cause slowing by direct action on the sinus node difficult to eliminate but so many reflex mechanisms, often antagonistic in their effect, could be concerned that it seemed hopeless to attempt the disentanglement of results obtained.

Since it is now reasonably certain that heart rate changes following variations of carotid pressure are chiefly mediated by reflexes involving the sinus caroticus nerve, the carotid artery toward the head was perfused with a pulsating stream of defibrinated or heparinized blood at body temperature. The perfusion pressure could be varied either as regards mean pressure or pulse pressure. To do this a large stopcock mechanically opened and closed to varying degrees was interposed in a perfusion system, the pressure of which was controlled by a Mariotte system. By stopping the mechanism with the stopcock open, the vessel could also be perfused at different constant pressures between 30 and 200 mm. Hg.

To record the pressure fluctuations, two calibrated optical manometers of the Wiggers pattern were used, one to read the pressure pulses in the perfused carotid artery, the other, inserted well down in the same carotid, to register the arterial pressure pulses of the animal.

To eliminate the possibility that long continued perfusion augments the right auricular pressure and so produces either directly or indirectly changes in heart rate, the artery was perfused only for short intervals of time allowing time for the accommodation of any excess blood in the body reservoirs. However, the operation of such a mechanism would easily be recognized by changes in the arterial pressure pulses of the animal.

Results. Effects of changes in a constant perfusion pressure. The effects following a sudden alteration in a constant perfusion pressure were found to be identical with those reported by others (e.g., cf. Heymans, 1928). Attention must however be directed to several special points:

In the first place, it was discovered that when the cephalic end of the carotid artery is perfused with blood under a pressure that is constant in a perfusion system, such a constant pressure never exists in the vessel itself. As is shown, for example, in the upper record of figure 4, B, a considerable pressure pulse may exist even when a constant perfusion pressure of 112 mm. Hg is maintained in the supply bottle. This effect is apparently produced by the fact that the resistance in the many branches of the common carotid artery varies as the pressure in anastomatic vessels supplied by the heart of the animal rises and falls during systole and diastole. The magnitude of the pulsation varies inversely with the perfusion pressure; when this is low (40-50 mm.) it may average 40 per cent of the mean aortic pressure and at mean perfusion pressures above 200 or 250 becomes very small. It is clear that the results of all investigators who assumed they were increasing or decreasing a constant perfusion pressure are complicated by the fact that pulse pressure variations actually occurred. If pulse pressure variations be indeed important in investigating reflex changes in rate, we can account for a common observation, viz., that while a sudden increase in mean pressure produces an undoubted reflex slowing, a sudden diminution often causes no changes in rate or a comparatively slight acceleration after a considerable delay. In other words, as in experiments with aortic leaks a sudden fall of pressure accompanied by increased pulse pressure appears to be without any great effect. The possibility thus grows that the reflex accelerating effect created by fall of mean pressure might be neutralized or counterbalanced by the reflex inhibitory effect of a larger pulse pressure.

In the second place, attention may again be directed to the fact that when the perfusion pressure is suddenly increased, a pronounced slowing of the heart occurs at once, to be followed by a more moderate slowing. For example, in one experiment the cardiac cycle increased from 0.47 to 1.32 second in the second beat after perfusion with a "constant" pressure of 172 mm. was established. Thereafter the cycles progressively shortened until 6 seconds later a steady cycle length of 0.52 second was attained. Similar effects were obtained in shifting from a low to a higher pressure level. These observations are but confirmation of results obtained by others, but their significance has apparently not been stressed. They appear to show that a sudden elevation of pressure in the *sinus caroticus* is the more potent stimulus for inducing reflex slowing than the maintenance of a higher mean pressure level. Furthermore, since similar effects are likely to occur when the magnitude of pulse pressure variations

in the perfused carotid are artificially changed, it became obvious that in such experiments the rate changes during the steady state rather than those immediately following changes in pulse pressure must be used as an index of effects.

The effect of alterations in pulsating perfusion pressures. The stabilized changes in heart rate produced by altering the amplitude of pressure variations, with and without changes in mean pressure, must be discussed in relation to actual records. The segments of records in figure 4 are from an experiment in which the following procedures were carried out: 1. Carotid pressure was first recorded when the vessel was not perfused and subject only to the low, mean and pulse pressure variations maintained by collateral circulation (segment A). The heart rate was 144 per minute. 2. The cephalic end of the left carotid was perfused with a "constant" pressure which gave rise to spontaneous pressure variations equal to 14 mm. shown in the upper record of segment B. The actual mean pressure recorded equalled 97 mm. The lower curve shows a slight lengthening of the cardiac cycle equivalent on calculation to 4 beats per minute. 3. The perfusion pressure was discontinued and as shown in segment C, the heart rate was practically restored to that in segment A (144 per min.). 4. The cephalic end of the carotid was perfused with a mechanically interrupted pressure as shown in the upper curve of segment D. Systolic pressure equalled that of segment B, diastolic pressure was lower, increasing the pulse pressure to 33 mm. Mean pressure was calculated to be 87 mm. A slight lengthening of the cardiac cycle is evidenced by the lower carotid pressure pulse which, calculations showed, amounted to a decrease of 11 beats per minute. 5. Another period of nonperfusion was allowed to intervene and calculations from segment E showed that the heart had accelerated to 150 per minute. 6. The cephalic end of the carotid was perfused with a widely fluctuating pulsatile stream as shown by the large excursions in segment F. Calculations of this segment showed that mean pressure in the perfused vessel equals 74 mm. (i.e., 13 mm. less than in segment D) and the pulse pressure 96 mm. The heart rate decreased permanently to 130, i.e., 20 beats below the control of segment E. These experiments clearly show that a greater pulse pressure combined with a moderate decrease in mean pressure has a greater stimulating value than a somewhat higher mean pressure combined with a smaller pulse pressure (cf. segments B, D and F).

Figure 5 shows four portions of a record from another experiment. Segments A and B show the effect of perfusing the cephalic end of a carotid with an uninterrupted perfusion pressure. A is a control, B shows the stabilized effects during perfusion. Mean pressure in the latter is calculated to be 159 mm. or 58 mm. higher than the animal's own mean pressure. The pulse pressure variations due to collateral vascular effects is

only 5 mm. Calculations from the lower carotid curve showed a retardation of the heart by 17 beats or a 10.6 per cent decrease. This shows clearly that a permanent elevation of mean pressure with only small pressure variations is capable of causing a permanent cardiac slowing. In segment D (for which segment C is a control) the central end of the carotid was perfused with a pulsating stream. Despite the fact that the mean perfusion pressure is obviously much less and the pulse pressure much greater, the heart rate calculated from the lower curve decreased by 24

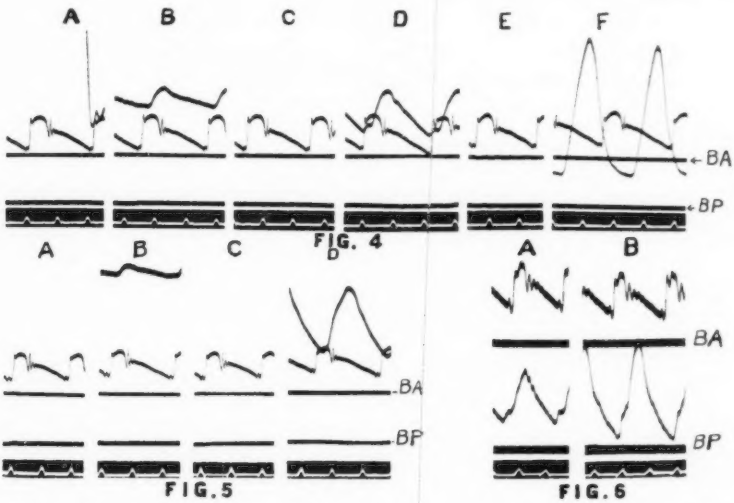

FIG. 4

FIG. 5

FIG. 6

Fig. 4. Experiment 26; 6EG. Optical pressure curves from left carotid illustrating the effect upon heart rate of perfusion of the carotid sinus with pulse pressures of varying magnitude and diminishing mean pressure. Segments A, C and E controls. B, perfusion with a constant pressure. D, and F, perfusion with an interrupted pressure. BA, base line arterial pressure. BP, base line perfusion pressure. Time 0.2 second.

Fig. 5. Experiment 26; 4EF. Same as figure 4. Segments A and C controls. B, perfusion under constant pressure. D, perfusion with interrupted pressure. Time 0.2 second.

Fig. 6. Experiment 30; 2GH. Same as figure 4. Showing the effect of increased pulse pressure when mean pressure remained constant. Time 0.2 second.

beats or 14.6 per cent. This demonstrates that in the same animal pulse pressure change is a more potent stimulus for production of reflex slowing than increase in mean pressure.

Figure 6 shows two segments of records in which the cephalic end of the carotid artery was perfused without alteration of mean pressure (132 $>$ 131 mm. Hg calculated) but the pulse pressure was mechanically increased

by 18 mm. The heart rate in segment A is 168 and in segment B, 161 per minute. The change is not marked, but distinct. The curve is published as an example of an experiment in which only minor variations occurred.

Similar results were obtained in many other experiments. These are summarized in condensed form in tables 2 and 3 in which the experiments are arranged in ascending order as regards the change in mean perfusion pressure. The other columns show the changes from the immediately preceding normal data. A glance at the results of table 2 shows that a decrease in cardiac rate by 7 beats or more occurred in two cases only (expts. 18–3,C,D and 20–7,E,F). Similarly the results of table 3 show a decreased heart rate in 10 experiments, no change or a reduction by less than 5 beats in 6 cases and an increase in 4 experiments. A careful study

TABLE 2

Perfusion of sinus caroticus. Change of perfusion mean − 2 to + 8. PP+

EXPERIMENT	PRESSURES IN MILLIMETERS OF MERCURY				HEART RATE CHANGE
	Perfusion		Arterial pressure		
	Mean	Pulse P	Mean	Pulse P	
18: 3AB	0	+54	−1	+2	−10
21: 6EF	0	+51	−9	+7	−16
16: 2AB	2	+42	+4	0	−15
28: 1CD	−1	+55	+5	+1	−12
29: 6AB	−1	+43	−2	+7	−7
30: 2AB	+1	+24	−8	−5	−7
18: 3CD	+1	+66	−4	−12	−4
26: 3CE	−2	+42	−6	+3	−7
26: 4FG	+3	+63	+2	+2	−10
26: 8FG	+8	+16	−5	+5	−9
20: 7EF	+6	−10	−5	−4	+3

of all the results composing this tabulation indicates that the heart rate changes obtained are generally accounted for by a summation of mean and pulse pressure effects in the perfused vessel; decreasing the mean pressure tending to cause a slight acceleration and a simultaneous increase in pulse pressure a more marked retardation. Only two experiments were found (nos. 24–3CD, 26–4BC) in which the rate changes are contrary to prediction according to this rule. One would scarcely anticipate cardiac slowing in experiment 30-2BC, since changes in mean and pulse pressure in the perfused artery were both slight and so nearly equal. Similarly, the tremendous decrease of mean pressure in experiment 26–3BC and 29–1AB may have prevented the dominance of the slowing action exerted by considerable increase in the pulse pressure.

It remains to direct attention to the slight or negative changes in arterial

mean and pulse pressure in the animals which are shown both in the published records and data of tables 2 and 3. This is important, for it forestalls a possible criterion that these changes may have modified the heart rate, through other reflex arcs. Any modifying effect they may have exerted is certainly limited to a very few of the listed experiments and does not invalidate the conclusion that changes in heart rate were chiefly due to pressure variations in the perfused carotid.

CONCLUSIONS. A consideration of all the experimental material allows the inference that changes in mean pressure of a perfused carotid artery—

TABLE 3

Perfusion of sinus caroticus, mean −6 to −51. PP+

EXPERIMENT NUMBER	PRESSURES IN MILLIMETERS OF MERCURY				HEART RATE CHANGE
	Perfusion pressures		Arterial pressures		
	Mean	Pulse P	Mean	Pulse P	
29: 3CD	−6	+41	−2	0	−6
26: 2AB	−8	+31	+1	−4	−9
30: 2BC	−9	+11	−2	−2	0
26: 5EF	−10	+23	+4	+2	−10
28: 1JK	−10	+19	−13	+4	0
22: 2AB	−15	+73	−4	+9	−16
28: 2EF	−17	+29	+3	−7	−8
26: 5CD	−17	+55	−3	+1	−4
28: 1IJ	−21	+58	−7	+4	−18
24: 3CD	−23	+55	−5	+12	+6
26: 4BD	−25	+45	−1	+2	+6
26: 6BD	−26	+78	0	−3	−2
29: 7CD	−27	+58	−6	+4	−17
26: 5GH	−30	+65	−4	0	−12
26: 3BC	−30	+32	+1	0	+8
20: 1AB	−31	+35	−11	+6	−6
26: 4EF	−36	+64	−2	+5	−13
29: 1AB	−41	+58	−4	+6	0
29: 5AB	−48	+75	−1	+4	−3
24: 2AB	−51	+55	+6	−4	+6

and more particularly an elevation of such pressure—is a factor modifying the heart rate reflexly. However, increase in pulse pressure when mean pressure is unaltered is a far more effective stimulus to the sinus caroticus endings, for in the great majority of experiments it dominates even when the mean pressure level is considerably decreased.

In view of these experiments, Marey's law seems to require amendment to the effect that reflex changes in heart rate are indeed induced by changes in mean pressure; but only so long as pulse pressure does not change in an opposite direction. When this occurs the changes of pulse pressure become supreme, in reflexly altering heart rate.

SUMMARY

Two sets of experiments with entirely different aims are reported: 1. In a first series of experiments the heart rate changes which occur reflexly or directly when pulse pressure and mean pressure were varied as independently as possible were studied. Aortic pressures were optically recorded and systolic, diastolic and pulse pressures calculated from records by use of a calibration scale. Mean pressure values were arrived at accurately by measuring the area beneath the pressure curve and dividing by horizontal distance.

a. The effects produced by various procedures such as compressing the vena cava and aorta served no useful purpose in settling the problem as pulse pressure and mean pressure changed in the same direction. They were useful in determining that under such conditions a mean pressure variation of 4 to 5 mm. is the minimal which causes any certain change in heart rate (e.g., 5 beats in 150 per min.).

b. After saline infusion which increases the pulse pressure, a cardiac slowing was obtained in a sufficient number of experiments in which no change or a decrease in mean pressure took place. These results were suggestive of a separate effect of pulse pressure, but in view of some apparently negative effects could not be regarded as conclusive.

c. Through the expedient of producing experimental aortic insufficiency it was found possible to increase pulse pressure and simultaneously decrease mean pressure; further by compressing the thoracic aorta to a suitable extent, mean pressure was often restored approximately or exactly to normal, while pulse pressures increased still more. It was found that frequently the heart rate slowed despite a sharp decline of mean pressure and with one exception was always retarded when mean pressure was restored to normal levels by compression.

d. While the control of heart rate by pulse pressure changes was demonstrated, the results on the "whole animal" permitted no conclusions as to whether the effects were induced by direct or reflex action.

2. A second set of experiments was performed in order to determine whether changes of pulse pressures in a cephalic end of a perfused carotid artery can reflexly cause heart rate changes either when mean pressure remains unaltered or deviates in a reverse direction. To do this pressure pulses were recorded simultaneously from the perfused carotid and the animal's own artery by means of optical manometers.

a. Perfusion of the cephalic end of a carotid artery with a constant pressure in the perfusion system was found not to produce a constant pressure within the artery itself owing to effects of collateral circulations. The magnitude of the pulsation varied inversely as the perfusion pressure used, being larger when pressures were low and small when they were high.

Such dynamic effects complicate the interpretation of results of previous investigations.

b. A sudden change in pressure appeared more potent in producing temporary alterations in heart rate that permanent levels of pressure established.

c. Alteration of the perfusion pressures and rhythmical variations in such a way that pulse pressure increased in the perfused vessel while mean pressure remained unaltered or was even reduced were attended by cardiac slowing unless the reduction was too extreme. This demonstrates that pulse pressure variation dominates the production of reflex cardiac changes.

3. The conclusion is reached that Marey's law requires amendment; changes in mean pressure levels indeed control heart rate changes reflexly but only so long as pulse pressures do not change too much in an opposite direction. When this is the case the effects of pulse pressure changes dominate the reactions.

BIBLIOGRAPHY

ADRIAN, E. D. 1926. Journ. Physiol., lxix, 49.

BRONK, D. W. AND N. L. KALTREIDER. 1931. Proc. Soc. Exp. Biol. and Med., xxviii, 1014.

BRONK, D. W. AND G. STELLA. 1932. Proc. Soc. Exp. Biol. and Med., xxix, 443; Journ. Cell. and Comp. Physiol., xx, 113.

HEYMANS, C. 1928. This Journal, lxxxv, 498.

HEYMANS, C. AND J. J. BOUCKAERT. 1930. Compt. rend. Soc. Biol., ciii, 31.

PEARCY AND WEAVER. 1927. This Journal, lxxxii, 47.

WIGGERS, C. J. 1915. Arch. Int. Med., xvi, 132.

1928. Pressure pulses in the cardiovascular system. Longmans, Green & Co.

WIGGERS, C. J., H. THEISEN AND H. A. WILLIAMS. 1930. Journ. Clin. Invest., ix, 215.

POISEUILLE'S LAW AND THE CAPILLARY CIRCULATION

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The direct application of Poiseuille's law to the flow of blood through the capillary network meets with difficulties both theoretical and practical in nature. It is obvious that a law which holds for the movement of a homogeneous fluid through relatively large capillary tubes cannot be applied without further test to the movement of a suspension through minute capillary tubes whose diameters only slightly exceed the dimensions of the suspended particles. It is not possible, as yet, to estimate on theoretical grounds the relation between capillary pressure and the velocity of blood flow; this, as suggested by Hürthle (1919), could only be determined by simultaneous measurements of capillary pressure and flow.

In the experiments described in this paper a motion picture camera was used to record the flow of an opaque suspension through the capillary network of the frog's mesentery. The diameter, length and the rate of flow were determined for each vessel separately by studying the cinematographic film. On the assumption that in blood capillaries all deviations from the conditions of Poiseuille's law tend to increase the viscosity of the blood, the experiments indicated that the lowest possible pressure fall in the capillary network of the frog's mesentery is approximately 2 cm. water. This figure is probably considerably lower than the total theoretical pressure gradient and is definitely lower than the average gradient measured by a direct method (Landis, 1926). In addition, the experiments point out that, even when the capillary vessels themselves are used for such studies, the physical characteristics of the system make it impossible to use Poiseuille's equation except in a very limited sense.

METHOD. The experiments were carried out during the months of February and March on normal brown frogs (Rana temporaria). The animals were pithed and mounted on the frog board previously described (Landis, 1926). The lateral body wall was incised by means of a cautery and the mesentery was exposed on a glass stage. A constant flow of physiological salt solution kept the preparation cool and moist. The mesentery was illuminated by means of an arc lamp, the light being passed through a heat filter, a green screen and a substage condenser.

Intense illumination was used only while the cinematographic film was being exposed. In the intervals a thin sheet of white paper was placed between the arc lamp and the microscope to protect the mesentery from the injurious effects of excessive light.

The image produced by a low power objective and ocular was passed through a Zeiss Ikon "Microphot" apparatus. The preparation could be observed through the vertical section of the "Microphot" tube while through the horizontal section an image was focused on the 16 mm. film of a model 70 B Filmo camera. The camera was mounted on a separate stand to prevent vibration from being transmitted to the microscope. Extraneous light was excluded by fitting the aperture of the camera with a small black cylinder which was inserted within, but did not touch, a larger black cylinder on the horizontal tube of the "Microphot" apparatus.

Each frame of the cinematrograph film measured 10×7.5 mm. and the total magnification on the film amounted to approximately 4 diameters. The observed area of mesentery measured, therefore, 2.5×1.9 mm. This was large enough to include the capillaries arising from a single terminal arteriole along with most of the finest venous connections of these capillaries. The timing of the camera was tested by taking photographs of a running stop watch. Inspection of the negative showed that the error was less than 5 per cent.

Single corpuscles could be photographed only moderately well, and, at best, could be followed through only a few frames because in moving through the capillary they were in proper focus only for short periods of time. Therefore, a mixture of 3 per cent acacia and graphite, prepared according to the method of Drinker and Churchill (1927), was used to provide greater contrast.

A suitable area of the mesentery was chosen, and the camera was adjusted to the proper focus. A micro-pipette, having a diameter at the tip of 30 to 40μ, was filled with the filtered acacia and graphite mixture, and then placed in the micro-manipulator as described previously (Landis, 1926). The tip of the micro-pipette was introduced into the lumen of the artery supplying the area of the mesentery selected for photography. Ordinarily the intra-arterial injection was made near the base of the mesentery into one of the larger branches (diameter 200-300μ) of the mesenteric artery.

The camera was started and several seconds later the acacia and graphite mixture was expelled from the micro-pipette into the artery. To prevent admixture of blood and graphite in the area under observation it was necessary to continue the injection at a rate exceeding that at which graphite flowed into the capillary network. The graphite, therefore, filled the artery to the nearest proximal bifurcation where the excess was carried away, mixed with blood, into the adjacent capillary fields. Since the graphite passed through an orifice 30 to 40μ in diameter into a vessel

200 to 300μ in diameter it seems unlikely that this method of injection changed arterial pressure significantly, particularly since the injected artery was always in free communication with the remainder of the vascular system. Exposures were made at the rate of 32 or 40 per second until the blood vessels in the area under observation were filled with graphite.

A bromide enlargement, measuring 28 × 40 cm. was made from one of the photographs in which the vessels were completely filled with the graphite mixture. The radius of each capillary was measured at intervals of 1 cm. on this enlargement. The figures were averaged, divided by the magnification, and the radius of each length of vessel was then recorded

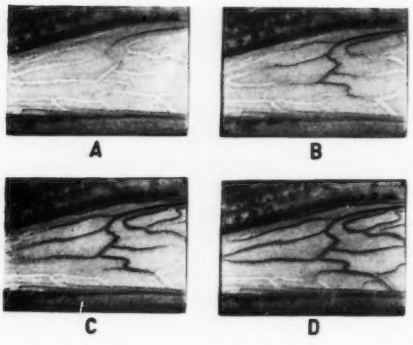

Fig. 1

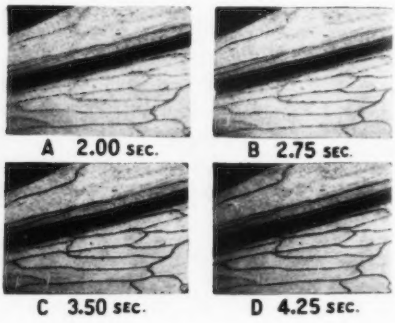

Fig. 2

Fig. 1. Cinematographic photographs showing the manner in which acacia and graphite mixture enters the capillary network. The exposures were made at the rate of 40 per second. Photograph A shows the graphite entering the arteriole. Photographs B, C, and D show the extent to which the graphite had penetrated the capillary network after 1, 2 and 3 seconds respectively. Magnification × 11.

Fig. 2. Cinematographic photographs showing graphite mixture following blood. The exposures were made at the rate of 32 per second. Photograph A shows the capillary network before the intra-arterial injection of graphite. Photographs B, C and D show the penetration of the graphite after 0.75, 1.50 and 2.25 seconds respectively. Magnification × 11.

in centimeters (tables 1 and 2). The length of each capillary was also measured on this enlargement.

Velocity of flow proved to be very difficult to measure on account of the manner in which the injected substance filled the capillary network. Figure 1 shows how 3 per cent acacia with graphite followed colorless 3 per cent acacia solution. In this case a cannula was introduced into the mesenteric artery and the mesenteric capillaries were perfused with the colorless acacia solution at a pressure of 40 cm. water. Acacia plus graphite was intro-

duced into the cannula and its flow through the capillary network was photographed at the rate of 40 frames per second. In entering the arteriole (fig. 1, A) the graphite was first visible as a tenuous spike the tip of which preceded a gradually widening zone of pigment. The graphite entered each capillary similarly (fig. 1, A, B, C) only gradually filling the entire lumen. When replacing blood, however, the tip of the stream of graphite was neither so distinct nor so long, as shown in figure 2, in which photograph A shows the capillary network before the graphite had reached the arterioles. In photograph B, exposed $\frac{3}{4}$ second or 24 frames later, the graphite had filled the arteriole to the right and had also advanced half way through the capillaries above the large vessel in the center. After another $\frac{3}{4}$ second, the capillary network was almost completely injected.

As may be observed in figure 2 the contrast between corpuscles and graphite was by no means so distinct as the contrast between colorless acacia and graphite. To determine the rate of flow the negative film was placed in a projector and each exposure was inspected on the screen as an image measuring about 1 meter in its larger diameter. The axial velocity in each capillary was determined by counting the number of frames exposed between the time at which the tip of the graphite column first entered the proximal end of each vessel segment and the time at which it reached the other extremity. This number divided by the number of frames exposed per second indicated the time required for blood to traverse each length of vessel. The length of the vessel in centimeters divided by the time in seconds gave the axial velocity in centimeters per second. Mean velocity was taken as half the axial velocity since in laminary movement of fluid the advancing particles distribute themselves in the form of a paraboloid (Hess, 1927a). Table 1 shows the mean velocities observed in the various segments of the network shown in figure 2. The numbers in the table refer to the vessels charted in figure 3 by which the individual vessel segments can be identified. Similarly the data in table 2 refer to the vessels charted in figure 4 and summarize the results of a second experiment in which graphite and acacia followed blood.

METHOD OF COMPUTATION. According to Poiseuille's law in the case of a liquid flowing through a long tube of small diameter the volume, Q, of liquid which escapes in time, t, is given by the equation

$$Q = \frac{\pi p r^4}{8 l \eta} t \tag{1}$$

where p is the fall of pressure in the tube, r the radius of the tube, and l its length, while η is the coefficient of viscosity. The volume, Q, escaping from a capillary can also be expressed by the equation,

$$Q = \pi r^2 V t \tag{2}$$

where V is the mean velocity per second. Substitution, rearrangement and changing the unit of pressure from dynes per square centimeter into centimeters of water yields

$$p = \frac{8l\eta V}{980r^2} \tag{3}$$

from which p, the pressure drop through length, l, may be computed from the radius, r, the mean velocity, V, and the coefficient of viscosity, η.

Poiseuille's law can only be applied accurately to the motion of true fluids as long as that motion is not turbulent. Reynolds (1883) developed a method of calculating the critical velocity below which fluid motion could not become turbulent. Applying Reynold's equation to the circulatory system led to the conclusion (Hess, 1927b) that even in the aorta turbulent motion is very unlikely to occur. The applicability of Poiseuille's law to a system of branching tubes has been affirmed by Schleier (1919) while Hürthle (1900) found that the law held also for a pulsating stream in capillary tubes between 0.5 and 1.0 mm. in diameter. Hess (1927b) in reviewing previous work concluded that Poiseuille's law probably holds for the flow of blood through vessels with a diameter of 0.1 mm. or more. Fåhraeus and Lindqvist (1931), however, reported a lowering of the apparent viscosity of oxalated blood as the size of the capillary decreased, probably associated with change in distribution of erythrocytes and plasma. The latter authors state that this can occur only where there is a definite axial stream and that the results of their investigation were "not able to elucidate the state of affairs in the capillaries themselves where the diameters of the corpuscles and vessels coincide."

In the capillary network itself all of the complicating factors so far investigated tend to elevate the effective viscosity of the blood above the viscosity as measured in relatively large glass capillary tubes. Obviously if a capillary is so small that it can barely accommodate one corpuscle the effective viscosity of the blood in that capillary will be greater than in larger vessels. When flow is completely stopped in a constricted capillary by one corpuscle this rise in viscosity must reach its maximum and the pressure drop will be maximal. Between this state of obstructed flow and the state of perfectly free flow there must be many gradations with corresponding differences in viscosity in individual capillaries. On the average the viscosity of the blood in the capillary network must, therefore, exceed the viscosity of blood as measured in the larger glass capillaries of the ordinary viscosimeter.

In addition, Poiseuille's law applies to the unrestricted smooth movement of concentric layers of fluid, the axial layers moving most rapidly, the peripheral layers moving most slowly. In the case of blood flowing through capillaries this type of flow is impossible since, if corpuscles almost fill the lumen, the fluid separating the corpuscles must be forced along in segments

rather than in a series of smoothly moving concentric layers. This type of movement is less efficient and would tend also to increase the viscosity figure.

Hess (1910), Rothmann (1913) and Rothlin (1920) showed that in a given capillary tube when the pressure fall was low the viscosity of blood was higher than in the same tubes when the pressure fall was greater. This is ascribed by Hess to the energy required to distort the blood in its movement. A column of blood flowing through capillary tubes is distorted whether the fall in pressure is high or low. It is only when the fall in pressure is low, however, that this factor destroys the relation between pressure fall and outflow which is expressed in Poiseuille's equation. The effect on viscosity of changing pressure fall was greater in narrow capillary tubes than in wider ones. In the capillary network the rate of flow is low, the pressure drop is relatively small, and the vessels are exceedingly minute. The conditions would all tend to increase the effective viscosity.

It is apparent from these findings that Poiseuille's law cannot be applied rigidly to any quantitative consideration of pressure drop in the capillary network. Since all the known conditions increase the effective blood viscosity by some unknown amount above the viscosity measured in larger tubes the formula can be used only to compute the lowest possible pressure drop that can be associated with observed rates of flow. The minimum values obtained in two capillary networks by the procedure described above seemed worthy of brief report, for comparison with the direct measurements of the pressure fall in the capillary network (Landis, 1926).

With the reservations mentioned above the equation used for calculation was

$$p = \frac{8l\eta V}{980r^2} \tag{4}$$

in which p, the pressure fall in centimeters water, was computed from the length of each segment of vessel in centimeters, the coefficient of viscosity of blood in dyne seconds or poises per square centimeter, the mean velocity of flow in centimeters per second, and the average radius in centimeters of each segment of vessel. The coefficient of viscosity of frog's blood at 20.0°C. is usually given as 0.0253 and similar figures were obtained by viscosimeter in two observations. This figure has been used in both series of computations reported below since the blood was being displaced from the capillary network while the observations were made. The coefficient of viscosity of the acacia and graphite mixture was 0.0175 dyne second per square centimeter.

Observations. The results of two experiments are given in tables 1 and 2, with accompanying figures 2, 3 and 4 to show the anatomical arrangement of the vessels studied. In figures 3 and 4 only those capillaries are

numbered which received their total blood supply from the single arteriole shown. In both instances the graphite mixture followed blood. The

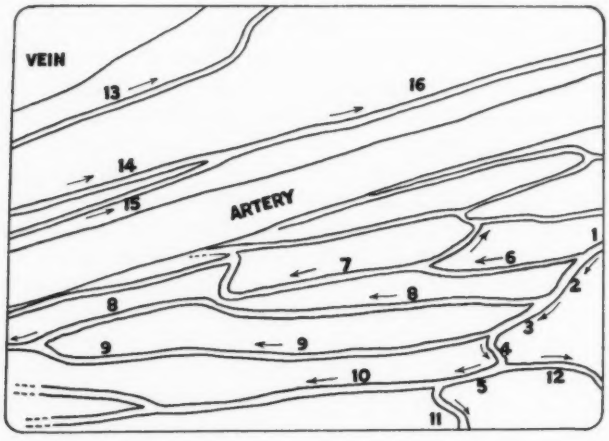

Fig. 3. Chart showing the arrangement of the arterioles and capillaries mentioned in table 1. The arrows indicate the direction of blood flow.

TABLE 1

Calculated pressure fall in vessels shown in figure 3

NUMBER OF VESSEL	TYPE OF VESSEL	RADIUS	LENGTH	MEAN VELOCITY OF FLOW	PRESSURE FALL	PRESSURE FALL PER MM. LENGTH
		cm.	*cm.*	*cm./sec.*	*cm. water*	*cm. water*
1	Arteriole	0.00146	0.012	0.389	0.5	4.2
2	Arteriole	0.00131	0.024	0.368	1.1	4.6
3	Arteriole	0.00137	0.024	0.368	1.0	4.2
4	Arteriole	0.00151	0.016	0.346	0.5	3.1
5	Arteriole	0.00134	0.024	0.240	0.7	2.9
6	Capillary	0.00116	0.066	0.078	0.8	1.2
7	Capillary	0.00146	0.100	0.101	1.0	1.0
8	Capillary	0.00146	0.206	0.102	2.0	1.0
9	Capillary	0.00126	0.184	0.069	1.7	0.9
10	Capillary	0.00120	0.114	0.044	0.7	0.6
11	Capillary	0.00121	0.026	0.120	0.4	1.5
12	Capillary	0.00125	0.044	0.055	0.3	0.7
13	Capillary	0.00113	0.130	0.045	1.0	0.8
14	Capillary	0.00165	0.094	0.043	0.3	0.3
15	Capillary	0.00167	0.093	0.051	0.4	0.4
16	Capillary	0.00231	0.160	0.058	0.4	0.3

admixture of graphite from two sources in a single capillary prevented the determination of velocity of flow.

The mean velocity of flow in the arterioles ranged from 0.75 to 3.89 mm. per second and the calculated pressure fall in the individual segments ranged from 0.3 cm. water in a length of 0.14 mm. to 1.1 cm. water in a length of 0.24 mm. The pressure fall per millimeter of arteriole ranged

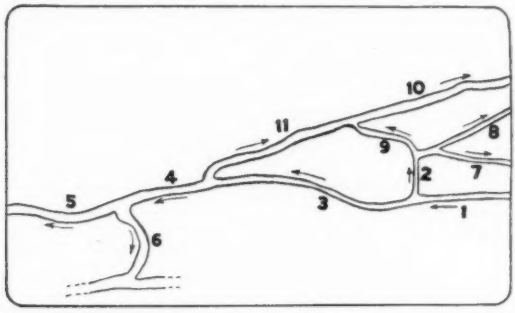

Fig. 4. Chart showing the arrangement of the arterioles and capillaries mentioned in table 2. The arrows indicate the direction of blood flow.

TABLE 2

Calculated pressure fall in vessels shown in figure 4

NUMBER OF VESSEL	TYPE OF VESSEL	RADIUS	LENGTH	MEAN VELOCITY OF FLOW	PRESSURE FALL	PRESSURE FALL PER MM. LENGTH
		cm.	*cm.*	*cm./sec.*	*cm. water*	*cm. water*
1	Arteriole	0.00113	0.034	0.140	0.8	2.3
2	Arteriole (?)	0.00081	0.014	0.075	0.3	2.1
3	Capillary	0.00119	0.068	0.095	0.9	1.3
4	Capillary	0.00150	0.034	0.037	0.1	0.3
5	Capillary	0.00165	0.040	0.026	0.1	0.2
6	Capillary	0.00133	0.024	0.037	0.1	0.4
7	Capillary	0.00086	0.028	0.044	0.3	1.1
8	Capillary	0.00070	0.031	0.031	0.4	1.3
9	Capillary	0.00070	0.027	0.043	0.5	1.8
10	Capillary	0.00139	0.059	0.039	0.3	0.5
11	Capillary	0.00125	0.055	0.031	0.2	0.4

from 2.1 to 4.6 cm. water. These figures cannot be regarded as more than approximate since the velocity of flow was so great that even though exposures were made at the rate of 32 per second the graphite advanced completely through certain of the shorter segments in the interval separating two successive photographs.

The mean velocity of flow in the capillaries ranged from 0.26 to 1.20

mm. per second and the calculated pressure fall for individual capillaries ranged from 0.1 cm. water in a length of 0.24 mm. to 2.0 cm. water in a length of 2.06 mm. The calculated fall of pressure per mm. of capillary varied between 0.2 and 1.8 cm. water. The results show the wide variation in rates of flow, and therefore in calculated fall of pressure in various capillaries arising from a single arteriole. In general, the very low pressure gradients were found in the larger venous capillaries, such as numbers *4*, *5* and *6* in figure 4 and *16* in figure 3, in which flow was sluggish and capillary diameter relatively large.

The dimensions and the pressure relationships of the arterioles and capillaries are summarized in table 3, which shows the average figures for the two vascular networks studied. The arteriolar segments were rela-

TABLE 3

Comparison of arterioles and capillaries

	ARTERIOLES	CAPILLARIES
Number of vessel segments studied	7	20
Average length	0.21 mm.	0.79 mm.
Average diameter	25.4 μ	20.6 μ
Average mean velocity	2.75 mm./sec.	0.57 mm./sec.
Average calculated fall of pressure per mm. length	3.3 cm. water	0.8 cm. water
Highest calculated fall of pressure per mm. length	4.6 cm. water	1.8 cm. water
Lowest calculated fall of pressure per mm. length	2.1 cm. water	0.2 cm. water

tively short, averaging 0.21 mm. in length, due to rapid ramification, as shown particularly in figures 2 and 3. The single capillary vessels were longer, averaging 0.79 mm. Actually, however, the total length of capillary network traversed by the blood was almost always between 1.0 and 2.0 mm. as may be observed in figures 3 and 4. In the network shown in figure 3 the blood traversed several short lengths, such as numbers *6* and *7*, or one longer length, such as *9*. In the network shown in figure 4 the blood from arteriole 2 traversed capillaries *9*, *11* and *4* before it reached a typical venous capillary, *5*. In the mesentery it is usual for the blood to pass through several capillaries in sequence and the total length of capillary traversed is therefore significantly greater than the average length of a single capillary.

The average fall of pressure in the arteriolar segments amounted to 3.3

cm. water per millimeter length of vessel due primarily to the very high velocity of flow. The arterioles studied in these experiments had an average diameter of 25.4μ. This is rather less than the average diameter (40–50μ) of the arterioles mentioned in an earlier study of the pressure gradient by direct micro-injection methods (Landis, 1926). In the observations described in the present paper the arterioles were terminal in location, and within the photographic field ramified into a number of true capillaries. The arterioles used for direct pressure measurements were more proximal in situation; they were not for the most part terminal arterioles. The difference in arteriolar diameter in these two sets of observations is due merely to the difference in the situation of the arterioles studied.

The average diameter of the capillaries was 20.6μ but this figure includes not only arteriolar capillaries but also the larger venous capillaries, a few of which measured well over 30μ. The fall in pressure in the capillaries was 0.8 cm. water per millimeter of capillary length. The total calculated

TABLE 4

Cross-sectional area of a terminal arteriole and its branches

	ARTERIOLES		CAPILLARIES		INCREASE IN CROSS-SECTIONAL AREA
	Diameter	Area	Number	Area	
	μ	μ^2		μ^2	
Arteriole 1	23	420	5	2410	6×
Arteriole 2	29	660	9	4220	6×
Arteriole 3	40	1260	13	6090	5×

fall of pressure in the capillary network of the frog's mesentery is in the neighborhood of 1.5 to 2.0 cm. water due to the fact that the blood traverses several short, or one very long capillary, as shown in figures 3 and 4. The figures are very variable, however, and differ widely in adjacent capillaries, due to individual differences in rate of blood flow.

It is of some interest to record the extent to which cross-sectional area changes as blood passes through the peripheral blood vessels. Table 4 gives the cross sectional area of a single arteriole and the sum of the cross sectional areas of all of the capillaries receiving blood from the arteriole. In three microscopic fields, measuring 2.5 × 1.9 mm., the total area of cross section increased by 5 or 6 times as the terminal arteriole ramified into its series of capillaries. This is a minimal figure since had the field been larger it is possible that a few more branchings might have been included.

Discussion. Mention has been made of the difficulties encountered in measuring the velocity of flow by the cinematographic method here described. The collective accuracy of the determinations of length, diameter and rate of flow was tested where possible by computing the

volume outflow of each vessel for comparison with the total outflow of all its branches. The figures usually agreed to within 10 to 15 per cent but occasionally larger errors amounting to 30 per cent were found. Therefore, the figures at best are only approximate.

The accuracy of the method can also be estimated by comparing the calculated drop in pressure in regions where blood may pass from one point to another by two routes. The pressure fall in these two alternative routes should be identical. In the network shown in figure 3 blood might pass from the junction of vessels *2* and *3* to the junction of vessels *8* and *9* by traversing a, capillary *8*, or b, arteriole segment *3* and capillary *9*. The calculated pressure fall in capillary *8* amounted to 2.0 cm. water compared to 2.7 cm. water, the calculated total fall in pressure in vessels *3* and *9*. The discrepancy is probably due chiefly to inaccuracy in estimating the velocity of blood flow in the arterioles, where the graphite advanced by relatively great distances between exposures.

In the capillary network shown in figure 4 blood might pass from the junction of vessels *1*, *2* and *3* to the junction of vessels *9*, *10* and *11* by traversing a, capillaries *3* and *11*, or b, arteriole *2* and capillary *9*. The calculated pressure fall in route a amounted to 1.1 cm. water compared to 0.8 cm. water in route b. The agreement here is slightly better probably because of the slower rates of flow.

The available evidence in the literature indicates that the effective viscosity of the blood is increased under the conditions existing in the capillary network. Poiseuille's law can therefore be used only to compute the lowest possible fall in capillary pressure. The two observations which have been described show that a pressure fall of a few millimeters of water in the capillary network could not produce the rates of blood flow observed in the mesentery of the frog. Apparently with average flow the fall in pressure in the capillary network *as a whole* cannot be less than 1 to 2 cm. water. By how much the true drop in pressure exceeds this figure it is at present impossible to calculate on theoretical grounds. It would seem that direct pressure measurements offer the only source of information.

The direct measurements of blood pressure in single capillaries by a micro-injection method (Landis, 1926) indicated that the pressure fall varies widely from capillary to capillary. The average fall of pressure in the capillary network amounted to 4.3 cm. water. The difference between the calculated and the observed figures is in accord with the original assumption that the viscosity of the blood is significantly greater in the capillary network than it is in the larger glass capillary tubes commonly used in viscosimeters.

SUMMARY

The diameter, length and rate of blood flow in the arterioles and capillaries of the frog's mesentery were determined by recording cinematographically the flow of an opaque suspension introduced by intra-arterial injection.

Evidence is given for the assumption that in blood capillaries the deviations from the conditions required by Poiseuille's law tend to increase the viscosity of the blood. Under these conditions Poiseuille's equation can be used only in the limited sense of indicating the lowest possible fall in pressure that can theoretically be associated with the observed rates of capillary blood flow. By how much the true fall in pressure exceeds this calculated figure must be determined by direct observations of capillary pressure.

The average calculated pressure fall per millimeter of length amounted to 3.3 cm. water in the arterioles and to 0.8 cm. water in the capillaries. The total fall of pressure in the entire capillary network apparently cannot be less than 1.0 to 2.0 cm. water.

A comparison of these calculated figures with the pressure fall determined directly by micro-injection methods indicates that the viscosity of blood is conspicuously increased by the conditions under which blood traverses the capillary network.

Poiseuille's law cannot be applied to the flow of blood through the capillary network except in the very restricted sense mentioned above.

I wish to express my gratitude to Prof. A. Krogh for many kind suggestions made during the course of this work.

BIBLIOGRAPHY

DRINKER, C. K. AND E. CHURCHILL. 1927. Proc. Royal Soc., ci, 463.
FÅHRAEUS, R. AND T. LINDQVIST. 1931. This Journal, xcvi, 562.
HESS, W. 1910. Zeitschr. klin. Med., lxxi, 421.
1927a. Handbuch d. norm. u. path. Physiologie, vii/2, 889.
1927b. Ibid., vii/2, 904.
HÜRTHLE, K. 1900. Pflüger's Arch., lxxxii, 415.
1919. Ibid., clxxiii, 158.
LANDIS, E. M. 1926. This Journal, lxxv, 548.
REYNOLDS, O. 1883. Phil. Trans., clxxiv, 935.
ROTHLIN, E. 1920. Zeitschr. f. klin. Med., lxxxix, 233.
ROTHMANN, M. 1913. Pflüger's Arch., clv, 318.
SCHLEIER, J. 1919. Pflüger's Arch., clxxiii, 172.

THE EFFECT OF ALTERNATING ELECTRICAL CURRENTS ON THE HEART[1]

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This paper constitutes a further report on our study of the effects of electrical currents on the dog heart as they relate to ventricular fibrillation (1). We were particularly interested in the suggestion made in the earlier literature (2) that ventricular fibrillation is not induced when a large current flows through the body and that if present can be stopped by a high voltage counter shock. We have found this statement to be true and further that a suitable electrical countershock may be used to resuscitate the fibrillating heart after electrical accidents. We have devoted our attention to the study of this question.

In addition to this material we have collected certain subsidiary data of interest to report. The subject matter will, therefore, be presented under several headings.

The present paper deals only with the effects of 60-cycle alternating currents upon dogs. The animals were always deeply anesthetized with morphine and ether.

High voltage circuits. Circuits carrying 1100 and 2200 volts were available. Contacts were made by means of electrodes soaked in saline solution and applied over shaved areas of the head and tail or hind legs.

In a series of seven experiments using the 2200 volt circuit currents of four or more amperes were passed through the animals many times for periods of from one-half to five seconds, ventricular fibrillation was never observed. In experiments with the thorax open violent muscular contractions dislocated the heart from the current path. In these latter cases, and in one experiment on an intact animal in which the initial current was in excess of five amperes and fell to zero during a shock of five seconds' duration because of a bad contact, ventricular fibrillation developed. It is believed that the resulting fibrillation in these cases was due to the fact that the amount of current passing through the heart was relatively small.

The contrast between high and low voltage currents is well shown in experiment 11/5/31. A current of 2.9 amperes at 1100 volts was passed

[1] Aided by a grant from the Physiological Committee of the Conference on Electric Shock.

through this animal twice for periods of two and five seconds without permanent effect upon the heart or respiratory center. Following this a 110 volt shock, the current being approximately 0.1 ampere, was given for two seconds. The peripheral pulse immediately disappeared and upon opening the thorax the ventricles were observed to be in fibrillation.

The effect of large currents is to cause the heart to cease to beat. After the circuit is broken the beats are promptly resumed, at first irregularly, but shortly with an apparently normal rhythm. In no case was the respiration permanently affected. One animal 11/3/31, exposed to two 2200 volt shocks, one for one-half and a second for one and a half seconds, was allowed to recover from the anesthetic. It showed no evidence of injury; when visited two days later it was eating and playing with other animals.

Amount of current requisite to fibrillate the ventricles. A surprisingly small amount of current is sufficient to throw the ventricles into fibrillation when suitably applied. In exploring this aspect of the problem we used special electrodes consisting of two needle points 3 mm. in length, separated 2 mm. from each other. The points were pressed into the musculature until the shoulders of the electrodes came into contact with the surface. The hearts, freed of the pericardium, were perfused in order to permit repeated tests since KCl can be readily employed to stop fibrillation and to obtain a normal preparation (3).

The feeblest current strength which our set-up permitted us to measure was one milliampere. In thirty-four applications of this stimulus to various areas on the external surface of two hearts we obtained permanent fibrillation ten times and transitory fibrillation five times. In earlier experiments (l.c.) we measured the amperage at the heart required to throw the ventricles into fibrillation when a current was passed from head to tail. In one case a heart current of 6 milliamperes caused fibrillation, the average of several determinations being 8.6 milliamperes. The difference between one milliampere and six milliamperes by the two methods seems to indicate that the concentration of current at any one point is the determining factor in the production of fibrillation.

The point of maximum irritability. Using the electrode and perfused heart described in the last section, we explored the accessible ventral surfaces of both ventricles and the internal surface of the right ventricle. The latter procedure was permitted by cutting the right ventricle along the septum except at the base and stopping the escape of perfusate with hemostats. The right ventricular mass thus separated continued to beat in unison with the rest of the heart.

In a single experiment, 4/12/32, a current of one milliampere was applied at different points of one- and two-second intervals forty-six times. Permanent fibrillation resulted eight times, three times with the one-second

application and five times with the two-second application. The points stimulated for one second followed by fibrillation were the extreme apex (once) and the middle region of the right side of the septum (twice). The points stimulated for two seconds followed by fibrillation were the extreme apex (twice), the middle region of the outer surface of the right ventricle (once) and the middle region of the right side of the septum (twice). Particular attention was paid to the septal surface in an effort to strike the the right Purkinje fiber bundle but no point could be found which was conspicuously more irritable than other neighboring points.

The results of this experiment, which were confirmed by another experiment with less refined technique, indicate that the extreme apex of the heart, which is presumably composed exclusively of muscular tissue, is as irritable and perhaps more irritable than any other region of the heart which was explored.

The countershock current. The foregoing section makes it clear that an extremely small current, of the order of one milliampere, may suffice to throw the ventricles into permanent fibrillation. On the other hand, the effect of strong currents is to cause a momentary rest of the organ like the compensatory pause which follows an extra systole. We have sought to define the effects of currents of intermediate strength when applied directly to the heart.

Most of our data was obtained upon the perfused heart but enough observations were made upon the heart with circulation intact to satisfy us that the isolated organ was an adequate preparation for the purposes in hand. The electrodes used were one inch brass discs, held securely against the sides of the exposed heart.

Currents up to 0.4 ampere, applied for five seconds, will cause fibrillation, and currents of 0.8 ampere or more will stop fibrillation. A current of 0.8 ampere will not cause fibrillation and in no case did a current of 0.45 ampere stop fibrillation. The only instance in which a current of 1.0 ampere failed to stop fibrillation was one in which the current strength was raised gradually from a fibrillating value of 0.1 ampere and held at 1.0 ampere for five seconds. In line with this observation was one in which a current of 1.0 ampere was applied to the beating heart for fifteen seconds and resulted in fibrillation.

It seems probable that the effectiveness of the countershock current depends to a degree upon the brevity of its application although five seconds is not excessively long. In such instances the fibrillation appears to continue, or to begin again if it has momentarily discontinued at first, and to stop only when the current is broken. A current of 1.0 ampere when applied for a tenth of a second is apparently just as effective as when applied for five seconds. This statement applies to a current path transversely with respect to the axis of the heart.

Experiment 4/5/32 was particularly directed to establish the effectiveness of the short-lasting countershock. In this case half-inch brass electrodes were used which maintained uniform contact with the ventricles by means of suitably adjusted spiral springs. A perfused heart was employed and the current strength was slowly and continuously raised from a fibrillation value to a countershock value on the one hand, and lowered from a countershock value to a fibrillation value on the other hand. In spite of the drawbacks inherent in this rather difficult procedure, the experiment convinced us that the short countershock was the most effective. The ventricles were thrown into fibrillation with a current of 0.8 milliampere and the current was gradually increased to 1.0 ampere without opening the circuit. The ventricles continued in fibrillation. Starting at the low value again the current had to be increased to 1.2 amperes before the fibrillation was arrested. The sixth time this test was made the current strength was gradually raised to 0.8 ampere and broken. Fibrillation continued. Shortly afterward a short countershock of this value, i.e., 0.8 ampere, promptly stopped the fibrillation and the heart resumed its normal rhythmic activity.

It was thus apparent that a higher amperage was required to stop fibrillation when the current strength was slowly increased than when it was suddenly and briefly applied. Decreasing the strength of the current from the countershock value did not produce such convincing results although we were left with the impression that the ventricles went into fibrillation with a current strength which would have been sufficient to stop the fibrillation, if applied suddenly to the fibrillating organ.

The countershock current applied to the intact animal. In applying countershock we have up to the present placed the electrodes on either side of the thorax in order to avoid passing the current through tissues which might be susceptible to injury. Under these conditions our experience indicates that electrodes of moderate size (2.75 sq. in.) are nearly as effective as larger ones. The simple ohms law relation does not hold for the passage of current through the body. There is a contact drop at each electrode which is practically independent of the size of the electrode. The resistance within the body is relatively small and decreases when larger electrodes are used due to the greater area of the current path. The evidence for these statements is presented in table 1, data for which were obtained in experiment 4/19/32. The study of table 1 shows that the total voltage drop was independent of the size of the electrodes and averaged approximately fifty volts for each electrode.

As already reported, a current of 0.8 to 1 ampere with the electrodes directly on the sides of the heart is sufficient to arrest fibrillation. However, in the case of the intact animal a much larger total current must be put through the animal in order to insure obtaining sufficient current

through the organ itself. In our experiments it was found necessary with the electrodes on either side of the thorax to use a current strength of approximately six or seven amperes to arrest fibrillation.

Recovery of the fibrillating heart and reëstablishment of the circulation following countershock. Our first experiments were made on preparations with the thorax open under artificial respiration, with the circulation intact and with the countershock electrodes applied directly to the heart. One ampere of current was usually employed for a very brief period, as has been indicated, to stop a previously established fibrillation. Our attention was first directed to the length of time a heart could be left in fibrillation and still recover a normal beat with reëstablished circulation after the countershock. It was found that such recovery could be obtained and obtained repeatedly on the same preparation if the duration of fibrillation did not

TABLE 1

ELECTRODE AREA	TOTAL APPLIED VOLTAGE	TOTAL CURRENT	CONTACT DROP	BODY RESISTANCE
sq. in.	*volts*	*amperes*	*volts*	*ohms*
2.75	124	0.8	105	29.0
2.75	150	3.0	110	35.0
2.75	194	4.8	110	18.0
2.75	215	5.7	78	20.5
2.75			Av. 101	Av. 25.6
13.0	120	1.2	107	16.6
13.0	194	8.0	103	12.0
13.0	180	7.5	93	11.0
13.0	145	5.6	89	8.3
13.0			Av. 98	Av. 12.0

exceed two minutes. If the ventricles were left in fibrillation for a period longer than two minutes they would develop feeble beats following the countershock but the force of the contractions was insufficient to reëstablish the circulation without recourse to other aids.

One of these aids was the central carotid injection of saline with adrenalin under a pressure of 150 mm. Hg as used by one of us for a similar purpose (3). The other aid was cardiac massage. Using these aids together after countershock, we succeeded in reëstablishing the circulation in one animal, 1/12/32, twice after five minutes and eight minutes of fibrillation.

In the paper last referred to fibrillation was overcome by the central carotid injection of 0.5 per cent KCl and it was there noted, as has been observed by D'Halluin (4) also, that attempt to facilitate a reëstablished circulation by cardiac massage was an unwise procedure since it was all too likely to throw the ventricles into fibrillation again. In this connection we

were impressed with the value of electric countershock because the heart did not show a conspicuous tendency to return to fibrillation when vigorously massaged.

Resuscitation experiments with countershock. Based on the foregoing observations the major portion of our attention was directed to resuscitating animals the hearts of which had been left in fibrillation for various lengths of time. In these cases the procedure was as follows: Under full morphia-ether anesthesia, the central end of the right carotid was cannulated for the injection of solutions and then electrodes, 2.75 sq. in. in area, covered with gauze wet with strong salt solution, were held firmly against the shaved areas on either side of the thorax with strong rubber bands. Ventricular fibrillation was established by giving a shock of a few seconds' duration using the 110 volt A.C. house circuit, after which a tracheal tube was inserted for intermittent insufflation. The countershock was applied with the same electrodes using sufficient voltage to send approximately six amperes through the animal. This was followed by central carotid injection of a salt solution containing adrenalin and heparin, saturated with oxygen and warmed to body temperature, under a pressure of 150 mm. Hg.

The three following protocols will serve as examples in detail of this procedure and of the type of results obtained.

3/29/32. Dog, male puppy, weight 10.5 pounds. Morphia-ether. The right foreleg and left hind leg were shaved for electrocardiograph leads. The sides of thorax were shaved and electrodes placed in position. The central end of right carotid was cannulated and connected with the pressure system for injection.

0 minute. House circuit applied for 3 seconds through thorax electrodes. Electrocardiogram taken. Ventricles in fibrillation.
2 minutes. Countershock, 6.7 amperes at 275 volts, for 0.8 second through thorax electrodes.
2 minutes, 10 seconds. Second countershock, 6.7 amperes for 0.1 second.
2 minutes, 30 seconds. Electrocardiogram taken. Weak ventricular activity recorded.
3 minutes, 30 seconds. Carotid injection (2 cc. adrenalin chloride 1:1000 in 75 cc. $CaCl_2$ 0.046 per cent in 0.9 per cent NaCl with heparin).
4 minutes. Pulse noted in femoral artery.
6 minutes. First respiratory effort noted. Pulse 200 per minute.
8 minutes. Pulse 80 per minute.
9 minutes. Eye-lid reflex obtained. Respirations regular.

Fibrillation was established and resuscitation obtained a second time on this animal, after which it was sacrificed before return of consciousness.

The accompanying figure (fig. 1) reproduces parts of the electrocardiogram obtained. 1—Gives the control cardiogram taken before the 110 volt shock; 2—shows the ventricles in fibrillation; 3—shows the ventricular pause which develops after countershock, together with the first evidence of ventricular activity before the solution was injected; 4—shows the

heart in process of recovery after the solution was injected when it was still in an anoxemic state, and 5—shows the heart well recovered.

3/18/32. Dog, full-grown female, weight 11.25 pounds. Morphia-ether. Thorax shaved and electrodes placed in position. Central end of right carotid cannulated and connected with pressure system for injection.

0 minute. House circuit applied for 5 seconds through thorax electrodes. No femoral pulse. Ventricles in fibrillation.
5 minutes. Countershock, 6.5 amperes at 275 volts for 0.85 second, through thorax electrodes.
6 minutes. Carotid injection (2 cc. adrenalin chloride 1:1000 in 50 cc. $CaCl_2$ 0.046 per cent in 0.9 per cent NaCl with heparin).
8 minutes. First respiratory gasp.

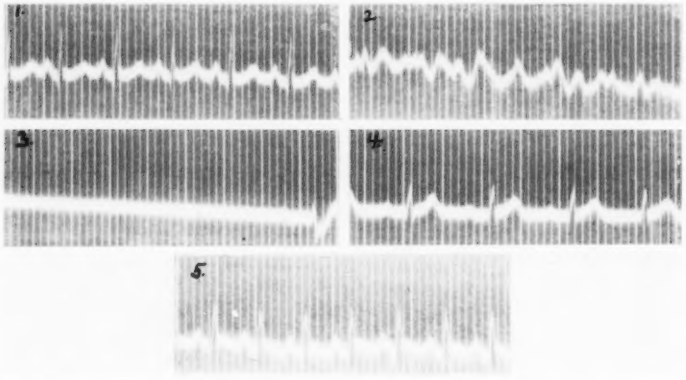

Fig. 1. Electrocardiograms obtained in experiment 3/29/32. This animal was left in fibrillation 2 minutes and two brief countershocks of 6.7 amperes at 275 volts were given. 1, reproduces the control cardiogram; 2, the ventricles in fibrillation; 3, the cardiac pause which succeeds countershock followed by the first evidence of spontaneous activity; 4, the heart in process of recovery after the solution was injected, and 5, the heart well recovered.

9 minutes. Pulse noted in femoral artery.
13 minutes. Pulse 140 per minute.
17 minutes. Eye-lid reflex obtained. Pulse 120 per minute.
20 minutes. Stopped intratracheal insufflation.
44 minutes. Pulse 84 per minute. Closed wound and returned animal to cage. The dog was found dead the next morning.

3/15/32. Dog, male puppy. Weight 10.75 pounds. Morphia-ether. Thorax was shaved and electrodes placed in position. Central end of right carotid cannulated and connected with pressure system for injection.

0 minute. House circuit applied for 5 seconds through thorax electrodes. No femoral pulse. Ventricles in fibrillation.

4 minutes. Countershock, 7 amperes (estimated) at 275 volts, for 0.7 second through thorax electrodes.
5 minutes. Carotid injection (2 cc. adrenalin chloride 1:1000 in 50 cc. $CaCl_2$ 0.046 per cent in 0.9 per cent NaCl, without heparin).
6 minutes. Pulse noted in femoral artery.
8 minutes. First respiratory gasp.
18 minutes. Pulse 48 per minute.
22 minutes. Pulse 80 per minute.
31 minutes. Eye-lid reflex obtained.
46 minutes. Pulse 72 per minute. Closed wound and returned animal to cage. This animal was in excellent condition the next day and showed no ill-effects subsequently.

TABLE 2

Showing failures to resuscitate after countershock

CASE	TIME IN FIBRILLATION	STRENGTH OF COUNTERSHOCK	DURATION OF COUNTERSHOCK	SOLUTION USED	CC./LB. INJECTED	CONDITION OF VENTRICLES	EXPLANATION
	minutes	*amperes*	*seconds*				
1	1½	9.0	2.00	$CaCl_2$, 0.023, NaCl 0.9%	10	Still	Blood clots in heart
2	1½	3.8	1.25	Ringer	9	Feeble beats	Blood clots in heart
3	3	6.4	0.90	$CaCl_2$, 0.046, NaCl 0.9, $NaHCO_3$ 0.02%; heparin	6	Fibrillation	None
4	3	6.2	0.90	$CaCl_2$, 0.046, NaCl 0.9, $NaHCO_3$ 0.02%; heparin	6	Still	None
5	4	9.0	1.00	$CaCl_2$, 0.023, NaCl 0.9%;	10	Feeble beats	Blood clots in heart
6	5	7.0	1.10	$CaCl_2$, 0.046, NaCl 0.9; heparin	10	Still	Distemper
7	5	3.6	1.10	Ringer	5	Feeble beats	None
8	5	3.7	1.40	Ringer	15	Fibrillation	None
9	5	7.2	1.40	Ringer; heparin	10	Fibrillation	None

The results of these resuscitation experiments are summarized in tables 2 and 3. Table 2 gives the nine cases which we failed to resuscitate. The first four columns give the case number, time during which the ventricles were left in fibrillation before countershock was applied, and the strength and duration of the countershock. The fifth and sixth columns give the solutions injected into the central end of the carotid and the number of cubic centimeters per pound injected. In each case 2 cc. adrenalin chlo-

ride, 1:1000, were injected at the same time. The seventh column indicates the condition of the ventricles when observed after failure of the procedure was established. The eighth column gives the explanation, if any, which was noted to account for the results.

Table 3 gives the thirteen cases of successful resuscitation. The first six columns present the data as described for table 2. The seventh (last)

TABLE 3

Showing successful resuscitations after countershock

CASE	TIME IN FIBRILLATION	STRENGTH OF COUNTER-SHOCK	DURATION OF COUNTER-SHOCK	SOLUTION USED	CC./LB. INJECTED	DEGREE OF RECOVERY
	minutes	*amperes*	*seconds*			
1	½	4.0	1.60	None	0	Permanent
2	2	4.0	1.00	Ringer	12	Failed after 30 minutes
3	5	6.5	1.25	Ringer	11	Transitory
4	2	6.2	1.10	$CaCl_2$, 0.046, NaCl 0.9, $NaHCO_3$ 0.02%; heparin	7	Failed after 15 minutes
5	2	6.7	0.80	$CaCl_2$, 0.046, NaCl 0.9%; heparin	7.5	Permanent
6	2	6.7	0.80	$CaCl_2$, 0.046, NaCl 0.9%; heparin	7.5	Permanent
7	2¼	6.7	1.25	Ringer; heparin	4	Permanent
8	3	6.5	1.00	Ringer; heparin	10	Permanent
9	4	7.3	1.25	Ringer; heparin	7	Failed after 7 minutes
10	4	8.0	1.25	Ringer; heparin	9	Failed after 19 minutes
11	4	6.7	1.25	$CaCl_2$, 0.046, NaCl 0.9%; heparin	5	Failed after 14 minutes
12	4	7.0	0.70	$CaCl_2$, 0.046, NaCl 0.9%; heparin	5	Permanent
13	5	6.5	0.85	$CaCl_2$, 0.046, NaCl 0.9%; heparin	7	Failed after 12 hours

column gives the degree of recovery observed. In only two of the six cases reported as having made a permanent recovery were the animals permitted to survive and return to consciousness in order to ascertain if any abnormality developed later as a consequence of the procedure. Both of these animals made an uneventful recovery. Attempt was also made to save case 13 but death occurred during the night. The other "perma-

nent" recoveries were sacrificed before return of consciousness but not until we were satisfied that complete recovery would have followed.

It will be noted that one animal (case 3) made a "transitory" recovery. By this we mean that a strong and regular femoral pulse developed subsequent to the procedure and then faded away for reasons unknown. Similarly there are five cases in which a vigorous peripheral pulse was recovered and which lasted from seven minutes to a half-hour. We have no explanation to offer for these partial successes unless it be that the resistance of the organism was insufficient to counteract the consequences of the period of circulatory rest. If such an explanation is accepted it is possible that more careful nursing together with adequate stimuli would have helped to carry over the period of depression.

The failures to resuscitate due to blood clots in the heart led us to use heparin in the injected fluid. While coagulation of the ventricular blood is not an invariable event it is apparently likely to develop, why we do not know. It is not related to the countershock because it was reported to be a disturbing factor in experiments, already referred to, in which the fibrillation was overcome by central carotid injection of potassium chloride (2).

Our experience has left us with the impression that the best solution for injection is one made up of $CaCl_2$ 0.046 per cent in NaCl 0.9 per cent containing a small amount of heparin. The excess of $CaCl_2$ and the absence of KCl as compared with Ringer solution does not conduce to a return of fibrillation after electrical countershock as it does under other circumstances (5) and gives another indication of the advantage of countershock in overcoming ventricular fibrillation.

This brings up again the issue of the necessity of central carotid injections to facilitate recovery after the fibrillation has been overcome by countershock. After short-lasting periods of fibrillation countershock alone will result in a reëstablished circulation. After longer periods of fibrillation, two minutes or more, the countershock is followed by feeble ventricular beats which are wholly inadequate to reactivate the circulation and we have thus far found no adequate measure for improving these beats other than central carotid injection of adrenalin and salt solution. The effect of this injection is to circulate a powerful cardiac stimulant in the coronaries and to fill the relaxed vascular bed so that when the ventricles do beat there will be sufficient peripheral resistance to continue the coronary flow. Then if the incoming blood from the lungs is adequate for the needs of the cardiac muscle a continuation of ventricular activity may be predicated.

SUMMARY AND CONCLUSIONS

Working with the dog heart in fully anesthetized animals, we have studied the effects produced by different values of 60 cycle alternating

current. This was used because of the ease with which permanent ventricular fibrillation may be established by electrical stimulation and because our main interest was to investigate the value of electric countershock in overcoming ventricular fibrillation.

Alternating currents of five or more amperes when passed through the body for one-half to five seconds will not produce ventricular fibrillation. However, the usual house circuit (110 volts) similarly applied will invariably produce fibrillation because the current that flows presumably is not sufficient to inhibit the heart.

One milliampere of current, applied directly to the ventricular musculature, is sufficient to cause fibrillation and the extreme ventricular apex is as sensitive as any other point on the ventricles.

With the electrodes applied directly to the heart, currents of 0.4 ampere for five seconds will cause fibrillation and currents of 0.8 ampere or more will stop fibrillation. A current of 0.8 ampere will not induce fibrillation and a current of 0.45 ampere will not stop fibrillation. In the intact animal with the electrodes on either side of the thorax the current spreads out over the body tissues. In order to obtain a sufficient current through the heart to arrest fibrillation, the countershock current must be increased to a value of at least four or five amperes.

Following the countershock the ventricles are quiescent for a brief period. When contractions begin they are very feeble but quickly increase in vigor and the circulation is reëstablished if fibrillation has not continued for long. If fibrillation has lasted for two minutes or more, spontaneous recovery of effective beats will not follow. Under these circumstances cardiac massage may be of signal benefit.

Of even greater assistance than cardiac massage is the central carotid injection of adrenalin in a salt solution. Our best results were obtained by injecting, under a pressure of 150 mm. Hg, from 5 cc. to 10 cc. per pound of a solution made up of $CaCl_2$ 0.046 per cent in NaCl 0.9 per cent containing heparin, saturated with oxygen and warmed to body temperature. To this mixture have been added 2 cc. adrenalin chloride 1:1000.

BIBLIOGRAPHY

(1) Kouwenhoven, Hooker and Langworthy: This Journal, 1932, c, 344.
(2) Provost and Battelli: Journ. Physiol. et Path. Gen., 1899, 1085, 1114.
(3) Hooker: This Journal, 1929, xci, 305.
(4) D'Halluin: Ann. de Soc. Sci. de Bruxelles, 1926, 1, 602.
(5) Hooker: This Journal, 1930, xc, 639.

A COMPARISON OF THE CALCIUM CONTENT OF HUMAN CEREBROSPINAL FLUID WITH THAT OF AN ULTRAFILTRATE OF SERUM

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Rona and Takahashi (1911) first showed that only a portion of the calcium of serum was capable of diffusing through a membrane. The hypothesis that variations in this fraction rather than in the total calcium of the blood may be important in influencing neuromuscular irritability has led to much interest in the question as to whether or not the calcium content of the spinal fluid represents the diffusible calcium of the blood. Discussion of this point in the literature has been based on several types of evidence but data actually comparing the calcium content of the spinal fluid of human subjects with that of the serum of simultaneously drawn blood and an ultrafiltrate from that serum are at present rather meager. (Kral, Stary and Winternitz, 1929; Greenberg, 1930; McCance and Watchorn, 1931.) In a recent publication Watchorn and McCance (1932) state, "Opinion is still divided as to whether ultrafiltration will wholly explain the calcium found in the cerebrospinal fluid."

In order to obtain "greater fluctuations of the blood constituents and thus a better chance to detect lags in the maintenance of equilibrium with spinal fluid" previous workers (Greenberg, 1930) have deliberately chosen pathological subjects.

We have determined the calcium content of the cerebrospinal fluid, serum and an ultrafiltrate of the serum in a series of eighty subjects. In all cases the values found were within the ranges usually accepted as normal. In view of the fact that the rather small number of determinations from which conclusions have been drawn have included chiefly pathological subjects it was felt that the presentation of our relatively large series might contribute to the knowledge of the normal relationship of the calcium of the spinal fluid to the diffusible calcium of the blood.

Our studies were made on patients at the State Psychopathic Hospital. The group includes fifty men and thirty women with an age distribution shown in table 1. Routinely the patients received a light breakfast and then were allowed no more food until after spinal fluid was obtained by lumbar puncture seven or eight hours later. Blood was drawn simultane-

ously and allowed to stand in the icebox overnight for the separation of the serum. Protein-free ultrafiltrates were obtained by the method of Greenberg and Gunther (1930), 3 or 4 cc. of serum being used for each determination. For the calcium determinations the method of Clark and Collip (1925) was followed, except that in all cases precipitation was allowed to continue overnight. Four cubic centimeters of spinal fluid were used in these determinations so that amounts of calcium and therefore the percentage errors involved should be comparable to those for 2 cc. of serum. The volumes of filtrate available were never more than 2 cc. and occasionally less than 0.5 cc. so that percentage errors due to over-stepping the end point are larger than in the case of serum or spinal fluid and this may account in part for the fact that the calcium concentrations of our filtrates

TABLE 1

Calcium of cerebrospinal fluid, serum and serum ultrafiltrate

All concentrations are expressed in milligrams per 100 cc.

AGE	NUMBER OF CASES	SERUM Ca		C.S.F. Ca		FILTRATE Ca		C.S.F. Ca / SERUM Ca × 100		FILTRATE Ca / C.S.F. Ca × 100		FILTRATE Ca / SERUM Ca × 100	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
years		*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*						
12–14	5	9.3–10.3	9.9	4.6–4.9	4.8	3.9–5.0	4.7	48–50	48	94–106	98	42–54	48
15–19	16	8.9–10.9	10.1	4.5–5.2	4.8	4.4–6.2	5.1	45–52	47	91–133	106	45–60	51
20–24	17	9.6–10.8	10.2	4.4–5.1	4.8	4.3–5.8	5.0	43–50	47	90–125	105	43–57	49
25–34	13	9.1–10.8	10.2	4.6–5.2	4.9	4.1–7.0	5.0	43–56	48	84–135	105	40–65	50
35–44	15	9.7–11.6	10.3	4.6–5.4	4.9	4.3–5.5	5.0	43–56	48	92–118	102	42–56	49
45–64	14	9.2–11.2	10.2	4.6–5.3	4.9	4.3–6.5	5.3	43–55	48	91–135	109	43–65	52
12–64	80	8.9–11.6	10.2	4.4–5.4	4.8	3.9–7.0	5.1	43–56	48	84–135	105	40–65	50
12–60	63*	8.9–11.2	10.2	4.4–5.4	4.8	3.9–6.2	5.0	43–56	48	84–133	104	40–61	50

* Selected group (all cases with organic brain or nervous disease omitted).

are in the majority of cases somewhat higher than those of the corresponding spinal fluids (table 1). All determinations were made in duplicate except in three or four instances when sufficient material was not available.

It seems well established that children tend to have a higher serum calcium concentration than adults. In view of the wide age range (twelve to sixty-four years) exhibited by our group, we have in table 1 shown the distribution of our results and certain ratios derived therefrom for several consecutive age groups. We are unable to see in these figures any evidence of the influence of age as such in our series.

In considering our data we compared our entire series of eighty-four determinations (with four subjects the determinations were repeated) with a smaller group of sixty-three obtained by omitting all subjects with

organic brain or nervous disease, toxic psychosis, positive Kahn test on the blood, and the few cases in which we had not been able to make duplicate determinations. However, as will be seen from table 1 the figures for ranges and means for all the quantities involved are practically identical in the two groups.

An inspection of the percentage ratios of our ultrafiltrate to spinal fluid calcium concentrations (table 1) shows that the range is surprisingly similar to those of the series of Greenberg (1930), 71 to 128 per cent, and McCance and Watchorn (1931), 79 to 133 per cent. For our entire series of eighty-four determinations 70 per cent of these ratios lie between 90 and 110 per cent. Greenberg (1930) has outlined some of the factors that might be expected to contribute to a difference between the calcium content of serum and lumbar spinal fluid even though there might be an actual condition of equilibrium between the diffusible calcium of the blood plasma in the capillaries of the choroid plexus and the spinal fluid in that region. Furthermore, if the differences obtained by subtracting the calcium concentration in milligrams per 100 cc. of each of our ultrafiltrates from the corresponding spinal fluid are plotted and the distribution compared with the normal probability curve of the same mean, -0.22, and standard deviation, 0.485, the fit is so close as to suggest strongly that the observed differences are due to experimental errors.[1] It would seem in any case that such frequent close agreement as we have found in our series can scarcely be dismissed as "merely fortuitous." In other words, our figures would appear to support the view that over the normal range of serum calcium values the spinal fluid calcium is a close measure of the diffusible calcium of the blood.

BIBLIOGRAPHY

Clark, E. P. and J. B. Collip. 1925. Journ. Biol. Chem., lxiii, 461.

Greenberg, D. M. 1930. Proc. Soc. Exp. Biol. and Med., xxvii, 514.

Greenberg, D. M. and L. Gunther. 1930. Journ. Biol. Chem., lxxxv, 491.

Kral, A., Z. Stary and R. Winternitz. 1929. Zeitschr. f. d. ges. Neurol. und Psychiat., cxxii, 308.

McCance, R. A. and E. Watchorn. 1931. Quart. Journ. Med., xxiv, 371.

Rona, P. and D. Takahashi. 1911. Biochem. Zeitschr., xxxi, 336.

Watchorn, E. and R. A. McCance. 1932. Biochem. Journ., xxvi, 54.

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A STUDY OF VITAMIN A DEFICIENCY IN NORMAL AND DEPANCREATIZED DOGS

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The symptoms of vitamin A deficiency, although in general typical, depend somewhat on the type and age of the animal used. The syndrome in young rats, described by observers (1), (2), (3), consists of loss of weight, loss of appetite, xerophthalmia and an unkempt appearance of the fur. Older rats are more resistant to the lack of the vitamin and the xerophthalmia may not occur, the animal dying of an intestinal complication or an intercurrent infection. In monkeys, Tilden and Miller (4) found that the intestinal symptoms, loss of appetite and listlessness developed first. Steenbock, Nelson and Hart (5) observed young dogs on an A deficient diet. The animals all gave signs of nutritive failure and three out of five dogs developed ophthalmia. The condition of the coat is not described but in the pictures of one of the dogs while A deficient, there is a definite thinning of the hair about the nose and around the eyes. The picture of the dog after its A deficiency was cured by cod liver oil shows the hair in these places to be quite normal.

We have observed in our laboratories for some years that the depancreatized dog kept on a diet of raw beef, raw pancreas, cracker meal and glucose, develops symptoms of vitamin A deficiency. These are loss of hair, scaliness of the skin, sores, running eyes and at times, a bloody diarrhea. Although this diet contains no large source of the vitamin, normal laboratory dogs may be kept on it for an indefinite period without showing any such symptoms. In reading the published protocols of other depancreatized dogs, one also finds references to these symptoms. Hédon (6) observed xerophthalmia, conjunctivitis and photophobia. Hershey and Soskin (7) and Best and Hershey (8) while observing the effect of lecithin in depancreatized dogs also noted symptoms of A deficiency. These investigators have shown that in such animals there is a disturbance of phospholipid metabolism which can be corrected by the addition of

[1] The expenses of this investigation have been defrayed by a fund from the Josiah Macy Jr. Foundation.

lecithin to the diet. They state that the effect of lecithin is not due to the presence of vitamins but rather to its ability to correct a condition which they describe as "liver failure" and which is characterized by jaundice bile in the urine and a decreased excretion of urine sugar. This disturbance of phospholipid metabolism is of particular interest because recent work in vitamin A has suggested some relationship between it and the phospholipins (9). Sherman and Boynton (10) have shown that nine-tenths of the total vitamin A in the body of the rat is in the liver. According to Leathes and Raper (11) the liver is the seat of phospholipid formation. In addition Olcott and McCann (12) have recently isolated from the liver of rats any enzyme carotenase which has the ability of converting carotene to vitamin A in vitro. We (15) were able to isolate this enzyme from the livers of normal dogs. In view of these observations and because of the symptoms of possible A deficiency in the depancreatized dog, it occurred to us that in such animals the metabolism of vitamin A might be altered. Several possibilities suggest themselves. One, that the liver is drained of its store of the vitamin more rapidly than normally, this depletion possibly resulting from a disturbance of the phospholipid metabolism. Or, secondly, that the enzyme carotenase might be suppressed or destroyed, thus impairing the ability of the animal to convert carotene to vitamin A. Finally, of course, the utilization of the vitamin may be interfered with.

PROCEDURE. Three groups of dogs, all females, were used. The first group (A, C and 12) were normal adult animals. Nos. A and C were on a mixed diet from the hospital kitchen. They were kept on this for ten months during which time they gained weight and appeared to be in excellent condition. No. 12 had been in our laboratories for eight months prior to the start of the experiment. Part of this time she received the mixed diet and for four months she received a diet of raw beef, cracker meal, lard and sugar. She was always in good condition and had gained 2½ kgm. during this time. She was placed on the A deficient diet, consisting of cracker meal 100 grams, sugar 30 grams, lard 50 grams, vitamins B, G and D in the form of irradiated yeast and 5 grams of a salt mixture containing two parts sodium chloride and three parts precipitated calcium phosphate. When the symptoms of A deficiency first developed the animal was given butter for 18 days. This was then discontinued and in 10 days the symptoms were again well developed. On February 6, 1932, 27 days after the butter was stopped, and when, as is seen in the protocol, the animal showed advanced symptoms of the deficiency carotene was started.

The second group (nos. 67, 68 and 69) were very young dogs. They were observed in order to follow the symptoms in young animals. No. 67 was a well nourished police puppy about 2½ months old at the start of the diet. Nos. 68 and 69 were about four months old and although very lively and in good condition, they were not as fat as no. 67. These dogs

received the same A deficient diet as dog 12, but in addition were given 100 cc. of boiled milk daily. When the symptoms of A deficiency were established dog 67 was given carotene. Dog 68 died on the 25th day of the diet; no. 69 was killed after 42 days of the diet.

The third group, the depancreatized dogs, consisted of six animals, nos. 51, 52, 54, 62, 63 and 66. All these dogs after operation were fed a diet consisting of raw beef, raw pancreas, cracker meal, sugar, irradiated yeast, and the salt mixture. Prior to operation dogs 62, 63 and 66 were on the A deficient diet for 26, 39 and 27 days respectively. Dogs 51, 52 and 54 were never on an A deficient diet and pre-operatively received raw beef, cracker meal and lard. After depancreatization, as soon as their wounds had healed, the dogs were allowed to become severely diabetic, receiving only small doses of insulin daily. They developed the symptoms of A deficiency as indicated in table 1. When the symptoms were well developed nos. 52, 62 and 63 were given carotene. This was obtained from the Mead Johnson Laboratories. It was put up in maize oil and had an antixerophthalmic potency ten times that of cod liver oil. We tested for the presence of vitamin A by the Carr and Price (14) modification of the Rosenheim and Drummond antimony trichloride test and the typical blue color due to vitamin A did not develop. The amount of carotene present in 1 cc. of the oil was estimated to be 1.32 mgm. To ascertain this the carotene was extracted by the method described by Palmer (15) and then compared to dichromate standards according to White and Gordon (16). All estimations were done in duplicate. The carotene was given about half an hour before the main meal with some glucose and raw pancreas and the dose of insulin was given at this time to insure the utilization of the carbohydrate. The dogs received the carotene as indicated in table 1. Dog 66 was given cod liver oil in tablet form 6 daily, and by injection, the latter was given for 7 days, 1 cc. daily. Both the tablets and ampoules were obtained from the White Laboratories. Each tablet contained 1,000 units of vitamin A. The dog received vitamin A in tablet form for 41 days.

That carotene is converted by the normal animal to vitamin A was shown by Moore (17) who fed it to young rats rendered A deficient. He found that 0.004 mgm. of carotene daily per rat was sufficient. After such feeding he found the predominant chromagen present in the liver was vitamin A.

In order to rule out the possibility of the symptoms being due to vitamin C deficiency, dogs 52, 63 and 67 were given 50 cc. of lemon juice for 9 to 13 days. This had no effect on the symptoms. Vitamin C deficiency is very difficult to produce in dogs, so that the possibility of its being a factor was remote.

Dog 51 was given two egg yolks during the time she was diabetic.

According to Sherman and Smith (1) "egg as a whole may be expected to contain about 15 to 20 units of vitamin A per gram; the yolk about three times this concentration." The yolk of an egg weighs about 20 grams (18). One can figure therefore that the dog received about 1200 units of vitamin A daily for 65 days. Egg yolks also contain lecithin and carotene. The amount of lecithin present in egg yolks varies considerably. According to Roaf and Edie (19) and Manasse (20) about 9.5 per cent of the yolk is lecithin. This means that at best, if a dog received 40 grams of egg yolk daily, it might be receiving about 4 grams of lecithin. Best and Hershey (8) gave their animals 10 grams of crude lecithin daily. Dog 54 received no pronounced source of the vitamin, being fed on the diet of raw beef, pancreas and cracker meal.

Technical procedures. All the animals but nos. 51 and 68 which were found dead, were killed by exsanguination. The liver was removed immediately and 40 grams used for the extraction of the enzyme carotenase. The method used in this procedure was a slight modification of that used by Olcott and McCann (12) and was reported previously (13). The criticism of our method of ascertaining the activity of the enzyme is that we depended on the development of a blue color with antimony trichloride to indicate the presence of vitamin A, rather than reading the band in the spectrophotometer. As the amount of the vitamin present from the conversion of the colloidal carotene is very small, this might be offered as an objection. However, as a control we also incubated colloidal carotene with a portion of the enzyme that had been rendered inactive by boiling. This invariably gave no blue color with antimony trichloride. The fatty acid content of the liver was done by the Mayer and Schaeffer modification of the Kumagawa and Suto method as described in *The Fats* by Leathes and Raper (11). The unsaponifiable matter was removed by the modified Lieberman method (11). The carotene and vitamin A content of the liver was read in the Lovibond tintometer. The method used for their extraction and determination was the Carr and Price modification of the Rosenheim and Drummond antimony trichloride test (14). Drummond and Hilditch (21) found that the agreement of intensities of the blue color as measured by the tintometer and the spectrophotometer was good. Realizing that the test, however, is subject to some variation by the individual worker all of these determinations were done and read by two members of the group. At least three readings were made on each liver oil. In addition to insure complete removal of bile pigments calcium hydroxide was added to the saponified liver.

Observations. Table 1 summarizes the observations on all the animals. In the normal animals loss of appetite and listlessness were the first symptoms, the change in the condition of the coat, loss of hair and sores appearing later in dogs 12 and 67. Dog 68 died before these symp-

TABLE 1

Summary of protocols

DOG NUMBER	DESCRIPTION	DIET PRIOR TO DEPANCREATIZATION	DIET AFTER DEPANCREATIZATION	PERIOD OF TIME DIABETIC	AVERAGE 24-HOUR EXCRETION OF SUGAR	ONSET OF A DEFICIENT SYMPTOMS	TYPE OF SYMPTOMS	AMOUNT OF CAROTENE RECEIVED	WEIGHT AT OPERATION	REMARKS
				days	*grams*				*kgm.*	
12 Control	Light colored; short haired	Not depancreatized A deficient diet with irradiated yeast 5 gm.				71 days after diet	Listlessness, loss of appetite, loss of hair, sores, loss from tail marked	57.5 cc. in 66 days		
67 Control	Police puppy	Not depancreatized A deficient diet with irradiated yeast 5 gm.				77 days, definite symptoms	Listlessness, shaggy coat, loss of hair, skin changes, cough and discharge from nose	33 cc. in 39 days		Received 50 cc. lemon juice for 9 days before carotene
51 Depancreatized	Tan and white; short-haired	Mixed normal	Raw beef 300 gm. Egg yolks 2 Sugar 50 gm. Pancreas 50 gm. Irradiated yeast 5 gm.	80	39	69 days after depancreatization	Loss of hair from face and legs. Sores, first on ears. Loss of hair from tail and body. Occult blood in stools. Loss of appetite	None	13 5	Received 2 egg yolks daily. Received lemon juice 90 cc. for 13 days
52 Depancreatized	Light colored; long-haired collie	Mixed normal	Raw beef 150–200 gm. Pancreas 100 gm. Cracker meal 200 gm. Sugar 50 gm. Lard 20 gm. Irradiated yeast 5 gm.	123	49	16 days after depancreatization	Sores at base of ears. Coat shaggy. Loss of hair in axilla and face. Skin reddened and crusty. Occult blood	Begun 24 days after symptoms. Received 50 cc. in 72 days	10½	Received cod liver oil for 23 days before operation. 50 cc. lemon juice for 13 days
54 Depancreatized	Black and tan-hound; short-haired	Mixed normal	Raw beef 75–200 gm. Cracker meal 250 gm. Sugar 50 gm. Raw pancreas 100 gm. Irradiated yeast 5 gm.	154	37	105 days after operation	Loss of hair from tail, and body. Sores over all joints. Loss of hair from face	None	10½	

			Irradiated yeast 5 gm.				hair from face			
62 Depancreatized	Black and white hound-like dog; hair about 1½ inches long	A deficient for 26 days preoperative	Raw beef 100 gm. Raw pancreas 100 gm. Cracker meal 75 gm. Sugar 75 gm. Irradiated yeast 5 gm.	75	28	About 15 days after operation	Loss of hair from face, then tail and body. Sores over joints and chest. Loss of hair from paws	34 cc. in 34 days	8½	
63 Depancreatized	Black and brown; hair slightly long	A deficient for 39 days preoperative	Raw beef 100 gm. Pancreas 100 gm. Cracker meal 100-50 gm. Sugar 75 gm. Irradiated yeast 5 gm.	73	17	About 25 days after operation	Sores on joints, loss of hair from head, face, tail, body	44 cc. in 44 days	8	50 cc. lemon juice for 8 days
66 Depancreatized	Light brown; short-haired; younger than other dogs	A deficient for 27 days preoperative	Raw beef 75-150 gm. Pancreas 50-100 gm. Sugar 40 gm. Irradiated yeast 5 gm.	62	6	Appeared mildly on day of operation; progressed rapidly after operation	Loss of hair from face, legs, paws, body and tail. Sores. Loss of pep	Received cod liver oil in tablet form 21 days after operation	6½	Given cod liver oil in tablet form and by hypodermic. Received lecithine for 7 days
68 Young dog; not depancreatized	Short haired; white and gray	A deficient diet; dog died of the deficiency				After 15 days	Loss of appetite, cough, listlessness, found dead in cage	None		
69 Young dog; not depancreatized	Short haired; tan and white	A deficient diet; killed on the 42nd day of the diet				After 28 days	Loss of appetite, very slight listness	None		

toms had an opportunity to develop; no. 69 was killed at the time she began to be listless, and no sores had appeared at that time.

The first definite sign of vitamin A deficiency in diabetic dogs is the change of the appearance of the animal's coat. This is accompanied or followed by loss of hair. The latter occurs in a fairly regular manner. Usually the hair is first lost about the nose, haunches and joints. Then the tip of the tail becomes hairless and about this time or sometimes earlier, the hair falls from the paws and the main portion of the body. Thinning of the hair around the margin of the eyes occurs late. The skin during this time becomes scaly and the surface flakes off. Underneath this the layer is reddened and the texture is quite different from normal. It feels rather like parchment. Sores appear practically everywhere—at the joints, near the front legs, on the chest, principally over all places where any pressure takes place. In the later stages there is a thin serous or bloody discharge from these sores. There is usually a non-purulent discharge from the eyes in the diabetic animals which is quite profuse. The stools are apt to be loose, sometimes watery and in the later stages fresh blood is present. We have not found the stools to be clay colored, but all of our animals received adequate amounts of raw pancreas.

Dog 12 overcame the symptoms of loss of appetite and listlessness 8 days after carotene was started; dog 67 improved after 4 days. Their coats showed marked improvement. The serous and blood tinged discharge from the sores stopped and the sores healed, although they broke down somewhat on no. 12 when the carotene was reduced. The three depancreatized dogs who were given carotene showed absolutely no improvement in the symptoms due to the loss of the vitamin. Their condition grew steadily worse, they were covered with sores and the hair was very sparse by the time the animals were killed. The two dogs who received no carotene showed no improvement in their symptoms at any time. The one dog who received cod liver oil in tablet form *did not overcome* the symptoms of vitamin A deficiency.

Table 2 shows the results of the analyses of the livers of the animals. The vitamin A content, reported in blue units, was highest in the two dogs who were never devitaminized and received a mixed diet. Dog 12, devitaminized and then fed carotene, had a much higher vitamin content than any of the depancreatized dogs. Of the depancreatized group the highest A content was in no. 52 who had received cod liver oil for 23 days pre-operatively. Even so, the blue units per 100 grams of liver were less than half of no. 12.

The carotene content, as reported in yellow units, was lower in all the depancreatized dogs with the exception of no. 51. This was the animal that received 2 egg yolks daily, which as mentioned before, means lecithin as well as vitamin A and carotene.

The test for carotenase was negative in three of the depancreatized dogs. It was faintly positive in one, and could not be done on the dog that was

TABLE 2

DOG NUMBER	CONDITION OF DOG	WEIGHT OF LIVER	BLUE UNITS PER 100 GRAMS	YELLOW UNITS PER 100 GRAMS	TOTAL BLUE UNITS IN LIVER	TOTAL YELLOW UNITS IN LIVER	BLUE TO YELLOW RATIO	FAT PER 100 GRAMS	CAROTENASE
		grams						*grams*	
12	Control devitaminized; given carotene	349	35,200	60	122,848	209.4	586.6:1	5.438	Markedly positive
A	Normal control; mixed diet	152.9	302,500	300	462,522	458.0	1,008:1	4.589	Positive
C	Normal control; mixed diet	543	132,000	150	716,760	815.0	880:1	1.838	Markedly positive
51	Depancreatized; meat and egg yolks	382	2,420	220	9,244	840.4	11:1		Not done; dog found dead
52	Depancreatized; carotene	553.1	14,000	79	77,434	439.7	176:1	2.792	Negative
54	Depancreatized; meat, cracker meal	341.8	4,840	60	16,543	205.0	80.6:1	2.743	Negative
62	Depancreatized; carotene	482	2,420	20	11,664	96.4	121:1	3.257	Positive
63	Depancreatized; carotene	340.5	220	30	749.1	102.2	7.3:1	2.690	Faintly positive
66	Depancreatized; cod liver oil	159	3,575	75	5,684	119.0	47.6:1	3.043	Negative
67	Young dog devitaminized; given carotene	224.3	1,760	50	3,914	111.0	35.2:1	2.706	Positive
68	Young dog; A deficient diet; died	107.8	*	10		10.8		Lost	Not done
69	Young dog; A deficient diet; killed on 42nd day of diet	122.5	100	75	122.5	91.9	1.3:1	0.334	Faintly positive

* Too low to estimate.

found dead. Of the other animals no. 69 showed a very faint positive test; in no. 68 the test could not be made, and the other dogs all showed a positive test.

The young dogs, nos. 67, 68 and 69, must be considered separately as young animals require more vitamin A and are more susceptible to a lack of it. Comparing these 3 young animals, no. 67 receiving carotene had 1,760 blue units per 100 grams of liver, which is striking in view of the fact that no. 69 only had 100 units per 100 grams and in no. 68, who died of the deficiency, it was impossible to make any reading as only the faintest blue tinge appeared. The carotene content in the young dogs was low.

Discussion. In the depancreatized dogs the vitamin A and carotene content of the livers is decreased. This occurred whether the animals received carotene or vitamin A or simply diet. The only exception is dog 51, who received 2 egg yolks daily, and the carotene content of whose liver was high. This might be interpreted to mean that some component of egg yolk, possibly lecithin, enables the animal to store carotene. If this were true the low carotene content in the other depancreatized animals may be due to a lack of storage. On the other hand the fact that the animal who did store carotene did not have a high vitamin A content would support the suggestion that the conversion of carotene is interfered with. If this interference with conversion is true it is probably a gradual process. We are carrying on experiments which we hope will clear up these points.

One fact is clear, that the vitamin A content in all the depancreatized dogs is low. This may be due to a loss of vitamin from the liver or to an inability of the animal to utilize the vitamin, or what is most probable, to a combination of these two functions. In view of the findings of Hershey and Soskin (7) that lecithin corrects the disturbance of fat metabolism observed in depancreatized dogs and because of the suggested relationship between vitamin A and the phospholipins, it is possible that this phospholipin is a factor in determining the ability of these animals to maintain a normal vitamin A metabolism.

Dog 12, rendered A deficient and then fed carotene, shows by the high vitamin and low carotene content of the liver, how rapidly the normal animal will convert carotene to vitamin A. That the carotene content of the liver was lower than in the two normal dogs, suggests that until the liver has stored a sufficient amount of vitamin A for the individual animal's needs, any precursor of the vitamin will be rapidly converted. This is also true in the group of young dogs, no. 67 who received carotene having a relatively high vitamin and low carotene content in the liver.

The question of the absorption of carotene by the depancreatized dog naturally presents itself. We endeavored to satisfy ourselves on this point by analyzing the stools of several of these animals for carotene, but we considered the observations unsatisfactory because of the difficulty of obtaining fresh stools. However, grossly there was no marked change in the color of the stools and the extractions as done showed only a small excretion.

There seems little doubt that the symptoms presented by these animals were due to a deficiency of vitamin A. The other vitamins B, G and D given in the form of 5 grams of irradiated yeast daily should take care of the animal's requirement. In addition the symptoms are not typical of the deficiency due to these vitamins. Sherman (22) has described these in rats and Cowgill (23) in dogs. In the latter the symptoms are loss of appetite, vomiting, polyneuritis and paralysis. Pictures of his dogs show *no loss of hair* and no sores.

CONCLUSIONS

1. Depancreatized dogs show symptoms of vitamin A deficiency.
2. These symptoms are not corrected by feeding the animals carotene. Vitamin A in the form of the concentrate and injections of cod liver oil did not cure the symptoms in one dog.
3. The vitamin A content of the livers of depancreatized dogs is low.
4. It is suggested that the failure to utilize vitamin A may be related to the disturbance of fat metabolism in these animals.

BIBLIOGRAPHY

(1) Sherman, H. C. and S. L. Smith. The vitamins. 1931, Amer. Chem. Soc. Mon. Series.
(2) Osborne, T. B. and L. B. Mendel. Journ. Biol. Chem., 1914, xvii, 401.
(3) Steenbock, H., M. T. Sell and M. V. Buell. Journ. Biol. Chem., 1921, xlvii, 89.
(4) Tilden, E. P. and E. G. Miller, Jr. Journ. Nutr., 1928, iii, 121.
(5) Steenbock, H., E. M. Nelson and E. B. Hart. This Journal, 1921, lvii, 14.
(6) Hédon. Journ. de Physiol. et de Path. Gen., 1927, xxvi.
(7) Hershey, J. M. and S. Soskin. This Journal, 1931, xcviii, 74.
(8) Best, C. H. and J. M. Hershey. Journ. Physiol., 1932, lxxv, 49.
(9) Monaghan, B. R. and O. Schmitt. Journ. Biol. Chem., 1932, xcvi, 2.
(10) Sherman, H. C. and L. C. Boynton. Journ. Amer. Chem. Soc., 1925, xlvii, 1646.
(11) Leathes, J. B. and H. S. Raper. The fats. Longmans, Green & Co., 1925.
(12) Olcott, H. S. and D. C. McCann. Journ. Biol. Chem., 1931, xciv, 185.
(13) Pariente, A. C. and E. P. Ralli. Proc. Soc. Exp. Biol. and Med., 1932, xxix, 1209.
(14) Carr, F. H. and E. A. Price. Biochem. Journ., 1926, xx, 497.
(15) Palmer, L. S. The carotinoids and related pigments. Amer. Chem. Soc. Monograph Series, 1922.
(16) White, F. and F. M. Gordon. Journ. Lab. Clin. Med., 1931, xvii.
(17) Moore, T. Biochem. Journ., 1930, xxiv, 692; Lancet, 1929, ii, 380.
(18) Mathews, A. P. Physiological chemistry. William Wood & Co., 1928.
(19) Roaf, H. E. and E. S. Edie. Preparation and estimation of lecithin. Thompson, Yates and Johnston, Lab. Rept., Liverpool, 1905, vi, 201.
(20) Manasse, A. Biochem. Zeitschr., 1906, i, 246.
(21) Drummond, J. C. and T. P. Hilditch. The relative value of cod liver oils from various sources. Empire Marketing Board. London, 1930.
(22) Sherman, H. C. Journ. Amer. Med. Assoc., 1931, xcvii, 1425.
(23) Cowgill, G. K. This Journal, 1921, lvi–lvii, 420.

THE NUTRITIVE DEFICIENCY OF MILK WITH SPECIFIC REFERENCE TO MANGANESE, ENERGY AND PITUITARY RELATIONS[1]

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That whole cow's milk is an unsatisfactory medium for the support of reproduction in the rat is a fact well recognized since 1920 (1) and 1922 (2) when Mattill and co-workers reviewed the literature and published their own observations. Mattill and Conklin (1) obtained no success by the addition of yeast extracts or iron citrate though growth was improved. In part the difficulty seemed to be the dilute form of the food because growth was better at first on milk powder, but again reproduction failed. The presence of a factor inhibitory for growth and reproduction was postulated, but not proven. Great variability in results with different animals was noted, which made the authors cautious about drawing conclusions.

Mattill and Stone (2) diluted milk powder with lard and starch and added salts. This allowed more normal growth at first, but reproduction again was not successful. It was noteworthy that reproduction capacity was permanently impaired. Additions of protein free milk, cod liver oil, traces of KI or even transfer to stock ration did not restore the reproductive function. Females ovulated freely on milk rations but males suffered degeneration of testes.

Palmer and Kennedy (3) also observed lack of fertility in females and even absence of mating instinct in males on milk rations. Their results were also variable.

Later Mattill, Carman and Clayton (4) acting on facts relating to requirements for vitamin E found extensive degeneration of the testes of males and resorption of embryos in females on a diet of milk powder supplemented with lard in addition to various other materials. Reproductive failure did not occur on these high fat rations after the addition of wheat embryo

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Anderegg and Nelson (5) made a very important contribution to our understanding of nutritive requirements when they pointed out that sometimes evidence for certain dietary deficiencies such as those relating to vitamin E, might actually be artifacts. Decomposition of fats when added to highly desiccated rations brought about resorption in utero in their experiments as though vitamin E were actually missing in the diet. Addition to the diet of water or ethyl alcohol or an anti-oxidant like wheat germ oil exerted a protective action.

Previously Anderegg (6) had found a diet which contained whole and skimmed milk powder as the sole source of protein and vitamins satisfactory for reproduction although the mortality of the young was high. He made various additions to milk powder, among which were casein, salts, iron citrate, agar, dextrin, starch, butter fat and lard, with the milk solids ranging from 50 to 99.8 per cent of the ration. In the latter case where 0.2 per cent of iron citrate was added, only 4 out of 15 young were raised by 4 females. He emphasized the importance of correct proportions of fat, protein, and salts in the diet.

Sure (7) pointed out that Mattill and co-workers (1), (2), (4) had not improved the protein moiety of their milk diets. Doing this by the addition of various proteins and amino acids did not, however, yield him success. Later he (8) made numerous multiple additions to milk powder and focused his attention on the resorption phenomenon in its relation to vitamin E.

Mattill and co-workers (9), (10), (11), (12), (13) have since developed the idea of destruction of vitamins,—which are and which are not concerned in reproduction, by oxidants and their protection by anti-oxidants in a most alluring manner; and Evans and Burr (14) have reported on the destructive action of certain fats. Evans and Bishop (15) reported that rats forced to subsist on fresh milk or whole milk powder gave evidence of infrequent occurrence of oestrum with delayed sexual maturation, although growth was not markedly depressed. No explanation for the abnormal performance was offered.

Daniels and Hutton (16) attempting to study the effect of heat sterilization of milk, found raw liquid milk unable to support reproduction in the rat except in rare instances. They found the multiple addition of manganese, fluorine, aluminum, sodium iodide, iron citrate, and sodium silicate as well as soy bean powder or its ash as satisfactory corrections.

Woods (17) found whole wheat an excellent supplement to milk, which she attributed mostly to an effect on the mineral metabolism. Ferric citrate alone, however, had little effect.

Mitchell and Schmidt (18) supplemented milk with manganese, fluorine, silicon, aluminum and iodine in the proportions used by Daniels and Hutton. Even though they left out the iron they obtained a decided improvement in reproduction, but did not draw any definite conclusions.

TABLE 1

Ovarian effect of pituitary transplants produced on different diets

	1	2	3	4	5	6	7	8	9	AVERAGE
Normal rats										
Milk + Cu and Fe:										
Total weight of 2 donors (♂), grams	630	600	452	524	530	518	518			539
Weight of ovaries of recipients, grams	0.015	0.025	0.022	0.016	0.014	0.016	0.014			0.017
Milk + Cu, Fe and Mn:										
Total weight of 2 donors (♂)	717	674	645	628	625	600	596			640
Weight of ovaries of recipients	0.015	0.011	0.020	0.017	0.015	0.019	0.011			0.015
Stock ration:										
Total weight of 2 donors (♂)	657		741	699	719	701	683			700
Weight of ovaries of recipients	0.016		0.015	0.016	0.015	0.014	0.015			0.015
Stock ration + 0.01% Mn:										
Total weight of 2 donors (♂)	660	660	749	700	710	701	683			695
Weight of ovaries of recipients	0.014	0.015	0.016	0.013	0.016	0.016	0.016			0.015
Stock ration but no H_2O:										
Total weight of 2 donors (♂)	475	475	475	445	445	441	368	372	378	430
Weight of ovaries of recipients	0.016	0.015	0.015	0.019	0.015	0.015	0.015	0.015	0.021	0.016
Castrated rats										
Milk + Cu and Fe:										
Weight of one donor (♂)†	270	230	297	263	345					279
Weight of ovaries of recipients	0.021	0.054	0.036	0.033	0.042					0.039

Milk + Cu, Fe and Mn:										
Weight of one donor (♂)†	316	328	340	328	318					326
Weight of ovaries of recipients	0.026	0.030	0.019	0.014	0.035					0.025
Stock ration:										
Total weight of 2 donors (♂)*	624	595	725	720	655	657				663
Weight of ovaries of recipients	0.138	0.062	0.026	0.044	0.064	0.046				0.064
Stock ration + high D:										
Total weight of 2 donors (♂)*	535	565	679	695	597	599				612
Weight of ovaries of recipients	0.125	0.024	0.037	0.053	0.093	0.039				0.062

* The first 4 animals were used for transplants 1 week after the feeding of high vitamin D was begun, the next 4, 2 weeks after and the last 4, 3 weeks after.

† These animals were used for transplants 14 weeks after castration.

Kraus (19) did not secure any reproduction on milk supplemented with iron and copper in spite of the fact, as stated by him, that males and females were kept together for long periods of time.

Keil and Nelson (20) in 5 months obtained one litter from 5 females receiving milk and iron, and from 4 to 5 litters each from 4 females receiving copper in addition. They state that they cannot explain Kraus' results nor those of Daniels and Hutton.

In a previous publication (21) we showed that fresh whole milk supplemented with iron and copper to prevent the development of anemia was still an inadequate diet for normal reproduction. Pregnancies were few, young were raised with difficulty if at all, the oestrous cycles were infrequent, irregular in length, and the opening of the vagina was delayed. However, 3 rats when given Mn, I, or both, in addition to Fe and Cu, came into oestrum with cycles of approximately the normal length, and other rats given a higher solids intake, by adding milk powder, showed more rapid growth.

Recently Skinner, Van Donk and Steenbock (22) have reported normal oestrum on a milk-Cu-Fe diet after the addition of sucrose and manganese and an improved oestrum with the addition of either alone.

Scott and Evans (23) in an attempt to determine the cause for variation in oestrum compared their table scrap stock ration with a diet of whole wheat, casein, whole milk powder, salt, calcium carbonate and butter fat for efficiency in supporting a normal rhythm in the rat. Because sexual activity was approximately the same with irregularities and infrequency of function in some animals on both diets, they concluded that nutrition was not necessarily the controlling factor. In support of this we have the pioneering observations of Smith and co-workers (24), (25), (26) and Zondek and co-workers (27), (28),—which have been confirmed repeatedly, to the effect that the oestrous cycle is controlled by the anterior lobe of the pituitary. This naturally has led investigators to raise the question, if after all, dietary effects on oestrum may not be produced indirectly through the pituitary.

The technique of weighing ovaries after transplanting pituitaries into immature animals to measure ovarian activity as developed by Smith, Zondek and co-workers has provided one means of putting this to experimental test. Marrian and Parkes (29) transplanted pituitaries from vitamin B deficient rats into immature anoestrous rats and obtained a response of the same order as with pituitaries from normal rats. This made it seem probable that inhibition of oestrum was caused primarily by the anorexia. But Mason and Wolf (30) obtained a 43 per cent greater effect with pituitaries of rats on vitamin A deficient diets than with controls on normal diets. With castrated rats the increase was 100 per cent. Inanition was stated to reduce the potency of the pituitary.

In view of the afore-described results, we transplanted pituitaries from rats on various diets, and on which we had determined the length of cycles, into young immature females according to Smith and Engle's technique. We always used one or two males as the donors for each female recipient and made both transplants at the same time. In a few cases we used males which had been castrated because of the greater potency of their glands. The males were adults taken for the most part from stock. The milk fed males however were reared on their diets. The high vitamin D animals and controls were castrated at about 4 to 5 months of age. Vitamin D administration was begun 31 to 46 days after castration. The female recipients were approximately 25 days old and were taken from stock.

We compared our stock ration, milk-copper and iron ration, milk-copper-iron-manganese ration, stock ration plus additional manganese, stock ration plus high vitamin D and stock ration minus water. The copper, iron and manganese were fed in amounts of 0.5 mgm., 1.0 mgm., and 1.0 mgm. per 100 cc. of milk respectively. Water was withheld from the rats, where noted, for 14 days. During this time the rats lost, on the average, 142 grams in weight. The vitamin D additions, when made, amounted to 50 Steenbock rat units[3] per gram of ration. The rats consumed from 10 to 15 grams of ration daily.

Our results which are shown in detail in the attached table do not reveal any differences so far as they go. It is apparent that from the uncastrated animals the pituitary transplants were not potent enough in any case to disturb the normal balance. It is possible that if all our animals had been castrated differences might have been revealed, but it is noteworthy that manganese and high vitamin D produced no effect even in these. As high vitamin D inhibits oestrus and as Mn added to a milk-Cu-Fe diet facilitates it, it is evident that in these cases, at least, the pituitary was not affected.

As to the effect of various diets on the oestral rhythm, we have abundant evidence. Originally we observed that 9 out of 10 apparently normal virgin females which failed to ovulate frequently or completely on a stock ration of yellow corn 71.5, oil meal 15, butter fat 5, crude casein 5, alfalfa 2, sodium chloride 0.5, bone ash 1.0 and whole milk and water ad libitum promptly showed oestrum or increased their rate to the normal with a change of ration. This second ration consisted of the above dry stock ration 66 parts, whole milk powder 20, cod liver oil 1, yeast 5, egg yolk 5, dried beef liver 2, and wheat germ oil 1.

With milk we repeated the experience after our failure to correct the inability of milk to support normal reproduction by additions of iron and

[3] One Steenbock rat unit is that amount of vitamin D which will produce a narrow line of calcium deposits in the distal ends of radii and ulnae of standard rachitic rats on ration 2965 in 10 days.

Cu (21). We determined the effect of supplemental additions of whole milk powder, cream, and sucrose on the supposition that liquid milk might be too dilute a food for the rat. The resultant intake of an excess of liquid could cause difficulties in a number of ways. It could limit unduly the intake of solids by mere lack of capacity of the rat; diuresis could cause an undue washing out of indispensable constituents, and the warming up of large amounts of liquid would, of course, entail a further drain upon energy reserves. It will be recalled that the dilute character of milk as a food for reproduction has already been suggested by others (1), but in experiments recorded in the literature in reference to this no attention was paid to the prevention of the anemia which occurs on exclusive milk diets.

That restricted food intake does affect oestrum specifically is also already recorded in the literature. Papanicolau and Stockard (31) reported that underfeeding of guinea pigs led to a prolongation of the dioestrum. Evans and Bishop (15) found a pronounced effect of undernutrition produced by a restricted intake of a satisfactory diet. Rats kept at a weight of 60 to 85 grams failed to come into oestrum even once during a period of observation of 375 days; rats kept at 125 to 150 grams had their first oestrous cycle delayed until the 116th to 332nd day, and those kept at 200 grams until the 50th to 135th day of observation. Loeb (32) observed failure of development and even retrogression of ovarian follicles in guinea pigs underfed on their usual ration of grain, grass and vegetables.

For our experiments we used twenty-eight females weighing approximately 50 to 60 grams and 23 to 30 days of age divided into 4 groups. All groups were given whole milk plus 0.5 mgm. Cu as $CuSO_4$ and 1.0 mgm. Fe as $FeCl_3$ per 100 cc., but one group was given a further addition of 13 grams milk powder per 100 cc. thus almost doubling the solids content; another was given 48 cc. 19 per cent cream for each 52 cc. milk, and a third 17 grams sucrose per 100 cc. With sucrose and milk powder additions each 100 cc. of milk was increased to 115 cc. in volume. The animals were kept on galvanized iron wire screen bottoms and were weighed weekly. After the first four weeks vaginal smear examinations were made daily.

The addition of whole milk powder, and to a slightly lesser degree the addition of sucrose, improved growth considerably, sexual maturation was expedited and oestrous cycles were increased in number. The results with cream were distinctly unfavorable; growth and sexual maturation were actually inhibited; but when oestrum did occur, the frequency was apparently improved. However, due to variations with different animals, final conclusions in regard to this, i.e., for the cream, cannot be drawn. It is noteworthy that towards the end of the period of observation the average daily consumption on the milk powder supplement was 30 cc.; on sucrose 32 cc., and on cream 24 cc. In comparison with 40 cc., which

is a fair average intake for rats of this weight on whole milk plus Cu and Fe alone, it is seen that the rats on sucrose, for example, had their intake of milk solids reduced materially. This shows that when other conditions are favorable, the milk intake provided a fair margin of milk ingredients for normal maturation and oestrum.[4] After the experiment had run for 54 weeks we changed the animals which were on the milk-Cu-Fe diet to the modified stock ration previously described. The oestral cycles in the following 4 weeks promptly increased from 11 to 27 in number.

We have also carried out some experiments on the value of various supplements on pregnancy and the rearing of young. For this purpose we kept young vigorous females 4 to 6 months old in groups of 4 or more with males for 12 weeks. With a supplement of Cu and Fe alone we obtained only one litter which was not reared. The further addition of 1.0 mgm. of Mn per 100 cc. of milk made possible the production of 5 litters of which 3 were reared and further supplementation of 5 grams lettuce per rat per day also resulted in 5 litters. After the addition of Fe and Cu, supplements of 17 grams of sucrose, or 13 grams of milk powder +0.01 mgm. iodine produced no further increases in pregnancies, neither did 11 grams of milk powder plus 8 grams of beef liver without Mn. Outstanding was the fact that a supplement of Cu, Fe, Mn and milk powder produced the largest number of litters, viz., 7, of which 6 were reared. In a second series[5] of 4 females in each group receiving Cu and Fe a supplement of 10 grams sucrose per 100 cc. of milk gave 5 pregnancies from which only 1 litter was reared. Ten grams of sucrose per 100 cc. of milk plus Mn gave 5 pregnancies of which 1 litter was reared. Five grams of lettuce per rat per day gave 2 pregnancies of which 2 were reared and of 3.0 grams beef liver plus 2.0 grams yeast, 2.0 grams egg yolk, 0.5 gram cod liver oil and 0.5 gram wheat germ oil gave 6 pregnancies of which all litters were reared. This latter group together with the milk powder group of the previous series were the only groups fairly successful in rearing their young. Outside of Cu and Fe alone, the Mn + sucrose and the lettuce groups were the poorest for raising their young. As consumption records revealed that the total consumption of solids on the sucrose diets was greater than on the liver and milk powder diets and equal to that on the lettuce diet it is evident that the different results here were not due to a deficiency in energy intake. It also is noteworthy that the 3 young raised by 3 different mothers on the sucrose diet ranged in weight from 17 to 23 grams

[4] As commercial sucrose of the best quality carries some manganese it was considered possible that the improvement with its addition was the result of this contamination. However analyses revealed that the average daily intake of manganese on the milk sucrose diet was 0.0008 mgm. while on milk alone it was 0.001 mgm.

[5] For assistance in gathering these data we are indebted to Dr. J. T. Skinner.

while the young raised on all the other diets, which in some cases included 6 in a litter, weighed from 29 to 44 grams. While sucrose therefore facilitated oestrum, it was not satisfactory for lactation.

The effect of extreme deficiencies and excesses of different components of the diet has been given attention by various investigators. Hart, McCollum, Steenbock and Humphrey (33) reported interference with oestrus in cows restricted to an unfavorable diet built up from the wheat plant; a similar diet built up from the corn plant was reported to give normal results. Evans and Bishop (15) employed diets high and low in fats, carbohydrates and protein, and diets low in salts, vitamin A, and the vitamin B complex. On carbohydrate free diets oestrum was not interfered with, unless the protein was also low, when the high fat used in place of most of the protein disturbed palatability. The same result occurred when the protein was high and the carbohydrate low. Low fat interfered with oestrum on both high and low protein intakes, and so did low salts and poor protein such as occurs in the wheat kernel. Low vitamin A produced a desquamative change in the vaginal epithelium which made it difficult to measure the cycle because the smears had the same appearance as those obtained during the oestrous stage. Vitamin B deficiency rapidly caused inhibition of oestrum. But Marrian and Parkes (34) point out the complicating effect of inanition. They observed inhibition of oestrum in rats on a vitamin B deficient diet consisting of starch 73, casein 20, cod liver oil 2, and a salt mixture 5, but partial inanition adjusted to produce similar weight curves had a similar effect and refeeding caused a return of oestrum.

Wolfe (35) has reported an inhibition of oestrum in mice kept on a low salt diet. Goettsch (36) found no effect of deprivation of vitamin C in guinea pigs until they began to lose in weight. Burr and Burr (37) observed cessation of oestrum on diets which did not contain fatty acids. Vitamin E did not improve the irregularity when added to the low fat rations.

We made a few preliminary runs on the effect of specific dietary deficiencies on oestrous cycles in females raised to an age of 7 and 8 months on our stock diet. Some of them had raised more than one litter and all had been under observation for frequency of oestrum for at least 5 months. All had been found to come into oestrum consistently though at a rather slow rate. The diets were compounded to be low respectively in vitamin B, vitamins A and D, protein, salts and calcium. A few were high in vitamin B and calcium. The frequency in occurrence of oestrum was compared in periods of 6 weeks before with 6 weeks after the dietary change.

We confirmed the prompt cessation of appearance of oestrum with loss in weight on a diet low in vitamin B and low in protein. We also found an obscurance of the oestral changes in the vagina with the rats on a low vitamin A diet. But the other dietary abnormalities revealed nothing of importance during the time of observation. Oestrum appeared as before.

In view of the disappearance of oestrum with the anorexia on low vitamin B rations and an observation on the effect of I, we fed desiccated beef thyroid to some rats. Results with this were, however, not decisive. One gram of thyroid daily caused rapid loss of weight and failure to ovulate, with death resulting in 2 to 3 weeks. In one instance, where 25 mgm. were fed, cycles abnormally long were not decreased in length and in another, normal cycles were not lengthened. Even with 250 mgm. daily, which caused death in 5 weeks with rapid fluctuation in weight, the rat came into oestrum about 6 times. Its rate was not different from that before the change in diet.

This confirms the experience of Evans and Long (38) who, feeding fresh beef thyroid to rats, found little change in length of normal cycles unless an excessive amount was given when there occurred inhibition of oestrum with loss in weight. Even thyroidectomy did not prevent oestrum permanently. However Weichert (39) has reported a persistence of dioestrum after the feeding of desiccated thyroid to rats which had been showing a normal oestrous rhythm. Thyroid preparations differ in their toxic effects, but so far apparently a stimulating effect resulting in a shortening in the length of oestrous cycles has not been observed. It is even possible that in some cases the effect of manganese as observed by Waddell, Steenbock and Hart (21) and Kemmerer, Elvehjem and Hart (40) may be linked up with I and the thyroid. This is suggested by the fact that McCarrison (41) reported the prevention of thyroid hypertrophy in the rabbit on a cabbage diet with manganese or iodine additions. Orent and McCollum (42) however claim that rats grown to sexual maturity on Mn free diets had normal oestral cycles.

CONCLUSIONS

The capacity of pituitary transplants to stimulate ovarian development in rats was not increased by the addition of Mn to a milk-Cu-Fe diet.

Though hypervitaminosis D inhibits ovulation, pituitary transplants from male rats receiving an excessive amount of vitamin D were found equally potent with controls.

Deprivation of water did not produce a profound influence on pituitary potency; small differences may, however, have escaped detection.

Subnormal oestrum in rats raised on a successful stock rat ration was corrected in 9 out of 10 cases by improvement of the diet by the inclusion of liver, egg yolk, wheat germ oil, yeast and cod liver oil.

The supplementation of a milk-Cu-Fe diet with sucrose or milk solids improved growth, ovulation and reproduction in rats. A deficiency of milk in energy for reproduction is therefore suggested. When energy was supplied as sucrose the growth of young was inferior.

The feeding of desiccated thyroid did not stimulate the frequency of oestrum.

Oestrum was promptly inhibited by change to diets low in vitamin B or protein.

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BIBLIOGRAPHY

(1) Mattill, H. A. and R. E. Conklin. Journ. Biol. Chem., 1920, xliv, 137.
(2) Mattill, H. A. and N. C. Stone. Journ. Biol. Chem., 1922, lv, 443.
(3) Palmer, L. S. and C. Kennedy. Proc. Soc. Exper. Biol. and Med., 1922–1923, xx, 506.
(4) Mattill, H. A., J. S. Carman and M. M. Clayton. Journ. Biol. Chem., 1924, lxi, 729.
(5) Anderegg, L. T. and V. E. Nelson. Journ. Ind. and Eng. Chem., 1926, xviii, 420.
(6) Anderegg, L. T. Journ. Biol. Chem., 1924, lix, 587.
(7) Sure, B. Journ. Biol. Chem., 1923–1924, lviii, 681.
(8) Sure, B. Journ. Biol. Chem., 1923–1924, lviii, 693.
(9) Mattill, H. A. Journ. Amer. Med. Assoc., 1927, lxxxix, 1505.
(10) Mattill, H. A. Journ. Biol. Chem., 1931, xc, 141.
(11) Olcovich, H. S. and H. A. Mattill. Journ. Biol. Chem., 1931, xci, 105.
(12) Olcott, H. S. and H. A. Mattill. Journ. Biol. Chem., 1931, xciii, 59, 65.
(13) Cummings, M. G. and H. A. Mattill. Journ. Nutrition, 1930–1931, iii, 421.
(14) Evans, H. M. and G. O. Burr. Journ. Amer. Med. Assoc., 1927, lxxxviii, 1462.
(15) Evans, H. M. and K. S. Bishop. Journ. Met. Res., 1922, i, 335.
(16) Daniels, A. G. and M. H. Hutton. Journ. Biol. Chem., 1925, lxiii, 143.
(17) Woods, E. Journ. Biol. Chem., 1925, lxvi, 57.
(18) Mitchell, H. S. and G. Schmidt. Journ. Biol. Chem., 1926, lxx, 471.
(19) Kraus, W. E. Journ. Dairy Sci., 1929, xii, 242.
(20) Keil, H. L. and V. E. Nelson. Journ. Biol. Chem., 1931, xciii, 49.
(21) Waddell, J., H. Steenbock and E. B. Hart. Journ. Nutr., 1931, iv, 53.
(22) Skinner, J. T., E. C. Van Donk and H. Steenbock. This Journal, 1932, ci, 591.
(23) Scott, C. J. and H. M. Evans. Anat. Rec., 1921, xxi, 80.
(24) Smith, P. E. Proc. Soc. Exper. Biol. and Med., 1926, xxiv, 131.
(25) Smith, P. E. This Journal, 1927, lxxx, 114.
(26) Smith, P. E. and E. T. Engle. Amer. Journ. Anat., 1927, xl, 159.
(27) Zondek, B. and S. Ascheim. Zeitschr. f. Geburtsh. and Gynakol., 1926, xc, 273, 387.
(28) Zondek, B. and S. Ascheim. Klin. Wochenschr., 1927, vi, 248; Arch. Gynakol., 1927, cxxx, 1.
(29) Marrian, G. F. and A. S. Parkes. Proc. Roy. Soc. Series B, 1929, cv, 248.
(30) Mason, K. E. and J. M. Wolf. Anat. Rec., 1930, xlv, 232.
(31) Papanicolau, S. W. and C. R. Stockard. Proc. Soc. Exper. Biol. and Med., 1920, xvii, 143.
(32) Loeb, L. Science, 1917, xlv, 591; Journ. Amer. Med. Assoc., 1921, lxxvii, 1646.
(33) Hart, E. B., E. V. McCollum, H. Steenbock and G. C. Humphrey. Res. Bull., 17, 1911, Journ. Agric. Res., 1917, x, 175.
(34) Marrian, G. F. and A. S. Parkes. Proc. of the Royal Soc. B, 1929, cv, 248.
(35) Wolfe, J. M. Proc. Soc. Exper. Biol. and Med., 1929, xxvii, 323.

(36) Goettsch, M. Proc. Soc. Exper. Biol. and Med., 1929, xxvii, 71.
(37) Burr, G. O. and M. M. Burr. Journ. Biol. Chem., 1930, lxxxvi, 587.
(38) Evans, H. M. and J. A. Long. This Journal, 1931, xcvii, 61.
(39) Weichert, C. K. Physiol. Zool., 1930, iii, 461.
(40) Kemmerer, A. R., C. A. Elvehjem and E. B. Hart. Journ. Biol. Chem., 1931, xcii, 623.
(41) McCarrison, R. Indian Journ. Med. Res., 1931, xviii, 1311.
(42) Orent, E. R. and E. V. McCollum. Journ. Biol. Chem., 1931, xcii, 651.

IS THE EFFECT OF FLUORINE ON TEETH PRODUCED THROUGH THE PARATHYROID GLANDS?[1]

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Interest in the effects of fluorine ingestion has been stimulated by the observation that the feeding of rock phosphate to animals produces harmful results which are traceable to the presence of fluorine. Such effects as a disturbance of general health, impaired appetite, and more specifically faulty bone and tooth development, have been observed. No attempt will be made to review the literature in detail, as McClure and Mitchell (1) have recently presented an excellent review of this subject. However, especial reference will be made to certain experiments on the relation of fluorine to teeth which bear directly on the subject under investigation.

McCollum and co-workers (2) first observed the effect of fluorine upon the teeth of rats. These authors found that 0.05 per cent sodium fluoride produced an abnormal growth of the incisors, which appeared to be due to changes in structure and hardness, resulting in lack of natural wear. The upper incisors frequently grew in an arc of such curvature as to lead to penetration of the roof of the mouth.

Bergera (3) found that albino rats subjected to prolonged administration of sodium fluoride showed a retarded ossification of the connective cartilages and a greater transparency of bones to x-rays. The incisor teeth gradually lost their orange color, becoming at first more transparent and lighter, and later acquiring dark chocolate colored bands. The superior incisors increased in length while the inferior incisors diminished.

Smith, Lantz and Smith (4) (5) have found that water from St. David, Arizona, when reduced in volume and fed to rats, produced characteristic changes in teeth. Their experiments support the hypothesis that mottled enamel in human beings is due to excessive amounts of fluorine in the drinking water. It appears that the discoloration of the teeth in itself is

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a secondary phenomenon following the primary changes caused by the fluorine. It probably is dependent upon the character of the diet.

The literature reveals that there are other factors which have the same effect on growth of teeth as fluorine. For example, the effect of parathyroidectomy upon the rat has been reported from the laboratory of Weichselbaum, as a result of work by Erdheim (6) (7) (8) and Toyofuku (9). In addition to the appearance of tetany, the authors observed consistent effects on the incisor teeth, which correspond to changes induced by fluorine feeding. For example, there occurred loss of strength causing repeated fractures, loss of normal yellow color with the gradual assumption of an opaque and chalky appearance, and growth of the maxillary incisors in an arc of such curvature as to lead to penetration of the roof of the mouth. Following the work of these investigators, Hammett (10) made similar observations. With the removal of the parathyroids alone 20 out of 26 rats, or 76.9 per cent, were observed to have dental defects. When both thyroids and parathyroids were removed, only one out of 20 showed changes in the teeth.

In connection with the effects of parathyroid removal upon the teeth, it is of interest that changes in the parathyroids have been reported in conditions in which calcium metabolism was deranged. Bauer (11) observed enlargement of the parathyroids in osteomalacia. Erdheim (12) observed hyperplasia in the parathyroids of rats afflicted with spontaneous rickets. In these the nuclei of cells increased in size, and the protoplasm showed a greater affinity for eosin. Korenchevsky (13), following Erdheim's work, attempted to discover whether the removal of the parathyroid in normal rats on a normal diet causes rickets or osteomalacia as believed by Erdheim. He noted the gross changes in the incisors, after parathyroidectomy, as described by Erdheim and Toyofuku (6) (7) (8) (9), but did not confirm the results of Erdheim on the rickets producing effect of removal of the parathyroids. Pappenheimer and Minor (14) noted a marked increase in the number of cells in the parathyroids, but found no histological changes in the gland, in cases of human rickets. Luce (15) observed a marked enlargement due to hyperplasia of the gland in rats fed on a low calcium ration containing 2.5 per cent cod liver oil.

After our experiments were under way, we came across the work of Chaneles (16) who, in discussing the tooth changes in rats fed sodium fluoride, said that the results suggested either a fundamental disturbance in calcium metabolism, such as might be caused by an alteration in the organs that regulate it, especially the parathyroid, or some local disturbance in calcification. This author (17) studied histological sections of various organs, including the thyroid, but made no comment on the appearance of the parathyroids.

In view of the similarity in tooth changes produced in rats by parathy-

roidectomy and fluorine feeding, and of the frequent observation of parathyroid enlargement in deranged calcium metabolism, this study was undertaken to determine if the effect of fluorine is brought about indirectly through these glands. To put our observations on a fundamental basis with a certainty that we were actually dealing with a fluorine toxicosis we correlated them with observations on growth as obtained on high and low calcium rations with and without fluorine additions and with and without an excess of vitamin D.

Composition of rations. Rats of various sizes were fed sodium fluoride added respectively to a stock ration of average calcium content and to rations high and low in calcium. The sodium fluoride was fed at a level suitable for the production of a chronic toxicosis. Schultz and Lamb (18) found that young rats survived from 8 to 14 weeks on a ration containing as high as 0.25 per cent sodium fluoride. We first tried 0.3 per cent sodium fluoride added to a stock ration, with the expectation that young rats would survive 5 to 6 weeks. However, death occurred in 7 to 10 days. The sodium fluoride content of the ration was then reduced to 0.15 per cent. This level was used for all subsequent experiments which involved the use of a total of 304 animals.

In the first series of experiments, the basal ration consisted essentially of a stock rat ration. Specifically it was composed of yellow corn 71.5, linseed oil meal 15.0, crude casein 5.0, alfalfa 2.0, sodium clhoride 0.5, calcium phosphate 1.0, butter fat 21.0, skim milk powder 34.0, yeast[2] 3.0, cod liver oil 3.0. The sodium fluoride (c. p., Baker and Adamson) was added in the form of a 4 per cent solution in distilled water. This was evaporated on a part of the yellow corn which had previously been ground to a powder in a ball mill. The above ration was fed ad libitum to five young and to ten medium sized male rats weighing respectively 50 to 60 grams, and 120 to 175 grams. Littermates were used as controls. All rats were kept on wire screens and were weighed weekly. Distilled water was given ad libitum.

In the second series of experiments, the Steenbock and Black rachitogenic ration 2965 (19), consisting of yellow corn 76, wheat gluten 20, calcium carbonate 3, and sodium chloride 1, was used as the basal ration. It was fed to four groups of 10 rats each weighing from 54 to 63 grams. The basal ration was fed alone, and with additions of 0.1 per cent irradiated Fleischmann's dry yeast, 0.1 per cent irradiated Fleischmann's yeast + 0.15 per cent NaF and 0.15 per cent NaF alone. The irradiated yeast was used as a source of vitamin D. All animals in these groups were given one drop of crude carotin solution, equivalent to approximately 0.04 mgm. of carotin per week, to avoid a low intake of vitamin A. These rations were

[2] Obtained from the Northwestern Yeast Company, Chicago.

fed ad libitum, but a record was kept of the food consumption of individuals throughout the experimental period.

In the third series of experiments, a low calcium ration which was essentially a modification of the Steenbock and Black ration 2965 (19) was used as the basal ration. The ingredients of the basal ration were as follows: ground yellow corn 79, acetic acid extracted casein 16, yeast 3, sodium chloride 2. The rats were fed this ration with and without additions of 0.15 per cent NaF, 2 per cent cod liver oil and 0.15 per cent NaF + cod liver oil. The animals which did not receive cod liver oil were given 2 drops of crude carotin solution three times per week to insure a supply of vitamin A. This carotin solution was equivalent to approximately 0.04 mgm. of carotin per drop. After 4 weeks on the experiment, one drop of red palm oil three times per week was substituted for the carotin solution. These amounts of red palm oil and of carotin provided from 4 to 7 times the curative dose for vitamin A deficiency in young rats. Because of the early death of some animals in these groups, medium sized (103 to 174 grams) and large (210 to 270 grams) male rats were given the above modifications of the low calcium ration.

Observations on growth. A depressing effect of 0.15 per cent NaF in the stock ration on growth was pronounced in young animals kept on the ration for 15 weeks. At the end of the experiment, the control animals had increased in weight from an average of 55 up to 349 grams while the fluorine fed animals had increased their weight to only 133 grams. The medium sized animals which initially weighed 150 and 172 grams had lost respectively on an average 11 per cent and 3 per cent of their weight in 25 to 40 weeks, while those not receiving sodium fluoride more than doubled their weight in the same period. That these results were due, at least in part, to decreased food consumption was apparent from the fact that at the beginning of the experiment the fluorine fed groups ate only from 50 to 75 per cent as much as the controls.

We supplemented the above data on the stock ration with data of experiments on adult rats, which experiments were not provided for in our original plan. Five male rats weighing from 330 to 400 grams were fed alternately the stock ration and the stock ration plus 0.15 per cent sodium fluoride. With these the depressing effect of fluorine feeding was again demonstrated. During three weeks of fluorine feeding they lost on an average 75 grams in weight. Of this they regained an average of 60 grams when returned to the stock ration for two weeks, but lost again immediately when put back on the sodium fluoride ration.

The results obtained on the rachitogenic ration were in harmony with those obtained on the stock diet, as discussed above. Of the rats on this ration, the groups receiving sodium fluoride did not eat so well nor grow quite so rapidly as those receiving the rachitogenic ration alone. Started

at 56 to 57 grams those receiving NaF averaged 73 to 75 grams in weight after 6 weeks while the controls averaged 80 to 99 grams. The former ate 6.8 to 6.9 grams of food daily, the latter 7.3 to 8.5 grams.

We considered it important to determine whether the depression of body weight was due entirely to decreased food consumption or in part to a toxic effect of fluorine. For this purpose young male rats with and without NaF and with irradiated yeast and irradiated yeast plus NaF were paired on the low calcium and rachitogenic rations, and the food intake of controls and corresponding fluorine-fed rats was equalized for 42 days. At the end of this period it was noted that the animals receiving sodium fluoride weighed, on the average less than the controls.

These data support the findings of McClure and Mitchell (1) that sodium fluoride has a depressing effect on growth aside from its effect on food consumption. On the low calcium ration all of the rats receiving sodium fluoride weighed less than their controls. However, on our calcium rich rachitogenic ration plus sodium fluoride the difference in weight was not great, as among the 18 surviving animals 6 actually weighed from 2 to 7 grams more than the corresponding controls.

It is desired to emphasize particularly the increase in toxicity of sodium fluoride with decrease in calcium intake in both young and old rats. Out of a group of 10 young rats which were fed the low calcium ration plus sodium fluoride, only 6 survived for 4 weeks, 2 survived for 6 weeks, and all were dead within seven weeks after the experiment was initiated. In a corresponding group which had been fed the high calcium rachitogenic ration all survived the six weeks' experimental period. That this difference was not due to the basal ration is apparent from the fact that after six weeks on the low calcium ration, eight surviving animals weighed on the average, 90 grams, in comparison with an average weight of 80 grams for nine surviving animals on the rachitogenic ration alone.

In anticipation of an effect of the level of calcium intake on the toxicity of fluorine we included an addition of vitamin D in some of our experiments expecting that the toxic effect might thereby be reduced. As already stated, the vitamin D was provided in the form of irradiated yeast or cod liver oil. Two milligrams of the yeast and 75 mgm. of the cod liver oil provided not less than one Steenbock unit. Taking growth as an index the effect was beneficial on the low calcium ration but not on the high calcium rachitogenic ration. Evidently on the latter the excess of calcium in the diet had already dominated the situation.

Teeth. The effect of fluorine on the growth of incisor teeth as observed by others was confirmed. We were impressed with the increased antero-posterior diameter, particularly of the upper incisors, and with the bluntness of those incisors which did not break off. The overgrowth of the upper incisors as a result of fluorine feeding has usually been stressed.

Chaneles (22) mentions one rat in which the lower incisors grew at the expense of the uppers. In our animals, particularly in the older ones, the overgrowth of the lower incisors occurred almost as frequently as increased curvature of the uppers. When necessary we clipped both to prevent interference with eating, and penetration of the upper palate. In addition it was observed that the molars of those rats which were fed sodium fluoride for nine months were distinctly worn as compared with control animals. Such an effect on the teeth of cattle has been observed by Taylor (20) and by Huffman and Reed (21).

Parenthetically it may be stated that it was also observed that the nails, particularly those of the hind paws, became very long on those animals which were fed fluorine for several months. The difference was first noted after the animals had received fluorine for four months, but it became more pronounced as the animals grew older. After six months on the ration, all animals in the fluorine-fed group had longer nails than any of the animals in the control group. For example, at 9 months the big toe nails of 5 rats on the fluorine ration measured 7.8 mm.; on the control ration they measured 2.1 mm. The animals in the fluorine-fed group remained active until a day or two before death, therefore it seems improbable that the overgrowth of the nails was due to inactivity.

Parathyroids. Our studies of the parathyroids were confined to the main lobes. In the rat, the parathyroid gland consists essentially of two lobes, usually imbedded just beneath the capsule of the thyroid, on its upper border. Each lobe is roughly spherical or ellipsoidal in shape. Erdheim (6) and Toyofuku (9) have mentioned the occurrence of accessory parathyroids in the thymus gland, but state that these have practically no functional significance in the rat. Hammett and others whom he mentions (22) report that they have been unable to find accessory parathyroids in the rat. Hoskins and Chandler (23) reported accessory glands in 10 per cent of their rats. However, whatever the situation might be, it appeared that a study of the main lobes would meet our requirements. These were excised under ether anesthesia.

In the absence of available chemical or physiological methods for the study of parathyroid changes we were restricted to anatomical methods. The tissue was fixed in Bouin's solution, dehydrated and imbedded in paraffin. Serial sections were cut at 7 micra, and stained with hemotoxylin and eosin. The study of fixed tissue was considered justifiable because Luce (15) has shown that for purposes of comparison, measurements made after fixation are as satisfactory as those made on fresh material, although, of course, considerable shrinkage takes place on fixation. As a further precaution, fixation, imbedding, etc., were carried out by us according to a uniform technique. To make comparisons of size, two diameters, at right angles to each other, were measured on median sections, and the third

diameter was ascertained by multiplying the number of sections by the thickness in micra. The approximate volume was computed by means of a formula for determining the volume of an ellipsoid. In all, serial sections of 88 parathyroids were studied. The data are presented in table 1.

It was noted that on the stock ration and the low calcium ration, all diameters were less for the rats receiving sodium fluoride, with one exception.

TABLE 1

Size of parathyroids

SUPPLEMENT	NUMBER OF ANIMALS	AVERAGE WEIGHT OF RATS		DURATION OF EXPERIMENT	NUMBER OF GLANDS MEASURED	AVERAGE VOLUME
		Beginning	Final			
		grams	*grams*	*weeks*		*c.mm.*
Stock ration:						
None	5	55	349	15	3	0.141
0.15 per cent NaF	5	60	133	15	5	0.105
None	4	166	394	40	10	0.243
0.15 per cent NaF	3	172	157	40	8	0.151
Rachitogenic ration:						
None	9	57	80	6	6	0.059
0.15 per cent NaF	10	57	75	6	7	0.059
0.1 per cent irradiated yeast	10	56	99	6	5	0.053
0.1 per cent irradiated yeast + 0.15 per cent NaF	9	57	23	6	6	0.052
Low calcium ration:						
None*	4	61	76	4	4	0.167
0.15 per cent NaF*	4	63	58	4	4	0.182
C. L. O.	4	57	112	8	5	0.319
C. L. O. + 0.15 per cent NaF	5	56	72	8	5	0.181
None	6	113	154	6	2	0.283
0.15 per cent NaF	6	106	85	6	4	0.197
C. L. O.	6	128	218	6	2	0.171
C. L. O. + 0.15 per cent NaF	6	127	99	6	4	0.134
None	8	232	240	8	2	0.331
0.15 per cent NaF	8	235	168	8	2	0.278
C. L. O.	8	249	313	8	2	0.237
C. L. O. + 0.15 per cent NaF	8	252	199	8	2	0.146

* Food consumption approximately equalized.

This exception occurred in young rats whose food consumption was equalized. In these groups, the differences between corresponding diameters were much less, but the average volume of parathyroids of the fluorine-fed rats was slightly greater. On the rachitogenic ration, no clear-cut difference in size of parathyroid, either as between fluorine-fed and non-fluorine-fed, or as between rachitic and non-rachitic animals is seen. It appears that the severe rickets produced in young rats in six weeks on ration 2965

(19) is not accompanied by any such marked enlargement of the parathyroid as Erdheim (12) observed in spontaneous rickets. No influence of sodium fluoride on the size of parathyroids of rachitic rats was demonstrated.

In evaluating the apparently smaller size of parathyroids of rats fed sodium fluoride with the stock ration or the low calcium ration, one should remember that growth was markedly affected by feeding 0.15 per cent sodium fluoride, and that these differences may have been simply due to inhibition of growth. This seems probable since the marked difference in size disappeared when the food consumption was equalized in two groups on the low calcium ration. Hence our data do not prove that sodium fluoride influenced the size of parathyroids. It will be observed that the rats on the low calcium ration had larger parathyroids than much older animals on the stock ration, thus confirming the observations of Luce (15). Furthermore, it will be observed that rats receiving cod liver oil in addition to the low calcium ration had smaller parathyroids, although they grew better than those not receiving cod liver oil. Apparently the size of the parathyroids stood in inverse order to the amount of calcium available to the animal for its use.

We made a detailed examination to determine the presence of any structural changes in the parathyroids. Several variations in structure were observed. In many of the glands we noted the occurrence of smaller and more chromatic nuclei in cells similar to those described by Higgins and Sheard (24) as "regression of cell columns" or "shrinking of epithelial cords" in chick parathyroids. In some there occurred an increase in connective tissue stroma, greater vascularity, pyknotic nuclei, vacuolated cells and areas of apparent fatty degeneration. Of these variations, only one, viz., apparent fatty degeneration, was found exclusively in fluorine fed animals. But in all cases where it occurred it was restricted in area and in general the glands might be said to have an approximately normal appearance. In any event there was no abnormality present to a degree which would have suggested interference with functional activity of the gland as a whole. Parenthetically it may be stated that the kidneys of a majority of our rats which had received sodium fluoride for some months were pale and hobnailed, testicles showed atrophy of the germinal epithelium and almost complete absence of sperm, and small surface hemorrhages occurred in the pyloric mucosae.

CONCLUSIONS

We found no evidence that the parathyroid glands in the rat undergo any consistent significant change, either grossly or microscopically with the administration of toxic doses of sodium fluoride. Therefore the explanation for the apparently identical dental effects produced by sodium

fluoride administration and parathyroidectomy must be sought in some other mechanism unless in this respect anatomical criteria are of no value. The toxic effect of sodium fluoride administration as determined by growth was less on a high calcium ration than on a low calcium ration. On a low calcium ration the toxicity was reduced by the administration of vitamin D but on a high calcium ration this effect was not evident.

BIBLIOGRAPHY

(1) McClure, F. J. and H. H. Mitchell. Journ. Biol. Chem., 1931, xc, 297.
(2) McCollum, E. V., N. Simmonds, J. E. Becker and R. W. Bunting. Journ. Biol. Chem., 1925, lxiii, 553.
(3) Bergera, C. Rev. Soc. Argent. de Biol., 1927, iii, 303. From Physiol. Abstracts, 1927–28, xii, 462.
(4) Smith, M. C., E. M. Lantz and H. V. Smith. Univ. Arizona, Tech. Bull. 32, 1931.
(5) Smith, M. C., E. M. Lantz and H. V. Smith. Science, 1931, lxxiv, 244.
(6) Erdheim, J. Frankfürter Zeitschr. f. Path., 1911, vii, 175.
(7) Erdheim, J. Frankfürter Zeitschr. f. Path., 1911, vii, 238.
(8) Erdheim, J. Frankfürter Zeitschr. f. Path., 1911, vii, 295.
(9) Toyofuku, T. Frankfürter Zeitschr. f. Path., 1911, vii, 249.
(10) Hammett, F. S. This Journal, 1922, lxii, 197.
(11) Bauer, T. Frankfürter Zeitschr. f. Path., 1911, vii, 231.
(12) Erdheim, J. Rachitis und Epithelkörperchen. Wien, 1914 (quoted by Korenchevsky).
(13) Korenchevsky, V. Journ. Path. and Bact., 1922, xxv, 366.
(14) Pappenheimer, A. M. and J. Minor. Journ. Med. Res., 1920–1921, xlii, 391.
(15) Luce, E. M. Journ. Path. and Bact., 1923, xxvi, 200.
(16) Chaneles, J. Rev. Soc. Argent. de Biol., 1929, v, 317.
(17) Chaneles, J. Rev. Soc. Argent. de Biol., 1929, v, 386.
(18) Schultz, J. A. and A. R. Lamb. Science, 1925, lxi, 93.
(19) Steenbock, H. and A. Black. Journ. Biol. Chem., 1925, lxiv, 263.
(20) Taylor, G. E. Mich. Agric. Expt. Sta. Quart. Bull. 11, 1929, 101.
(21) Reed, O. E. and C. F. Huffman. Mich. Agric. Expt. Sta. Tech. Bull. 105, 1930.
(22) Hammett, F. S. This Journal, 1921, lvi, 196.
(23) Hoskins, M. M. and S. B. Chandler. Anat. Rec., 1925, xxx, 95.
(24) Higgins, G. M. and C. Sheard. This Journal, 1928, lxxxv, 299.

THE EFFECT OF THE LEVEL OF CALCIUM INTAKE ON THE CALCIFICATION OF BONES AND TEETH DURING FLUORINE TOXICOSIS[1]

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In the previous paper (1) we have called attention to the fact that the feeding of a toxic dose of sodium fluoride does not produce demonstrable histological changes in the parathyroid glands. We suspected that such changes might occur because parathyroidectomy and fluorine feeding both produce comparable modifications in the appearance of the dental structures accompanied by apparent changes in composition.

That fluorine feeding has an effect on bone calcification is a well known fact. Forbes (2) found a reduction in bone ash per cubic centimeter of volume in swine when they were fed rock phosphate which contains fluorine as a variable constituent. Tolle and Maynard (3) found a reduction of approximately 10 per cent in the bone ash of rats after 5 weeks on a ration in which the calcium supplement was supplied by 1.8 per cent rock phosphate. However, on a ration which contained 0.596 per cent calcium, McClure and Mitchell (4) obtained an increase of 1.3 per cent, although their balance experiments showed a decrease in calcium retention of 9.8 per cent. The ration which produced these results contained 0.06 per cent fluorine, which corresponds to approximately 0.13 per cent sodium fluoride. Chaneles (5) also found an increase in bone ash, amounting to about 5.5 per cent when sodium fluoride was fed in a basal ration of bread and milk but he found a 4 per cent decrease in the ash of teeth.

It is evident from these reports in the literature that the changes in composition of bone are variable. This suggested to us the desirability of securing further data particularly with reference to the effect of the basal diet. As calcium fluoride is relatively insoluble we surmised that the effect on calcification might stand in direct relation to the calcium content of the diet and therefore possibly to the facility with which calcium

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fluoride could be formed. An analysis of the results obtained with our diets respectively high, medium and low in calcium as used in the previous experiments was considered promising. Accordingly with the removal of the parathyroids from our animals we also dissected out tibias and femurs as well as the incisor teeth in the majority of cases. Molars were not removed because of the difficulty of securing them free from bone. With the expectancy that any changes observed might be correlatable with the composition of the blood, blood sera were analyzed for inorganic phosphorus according to the methods of Fiske and Subbarow (6). Determinations of serum calcium were made in duplicate on a protein-free filtrate obtained after treating the serum with four times its volume of trichloracetic acid. Aliquots corresponding to 1 cc. of serum were used. The hydrogen ion concentration was adjusted in the manner used by McCrudden (7) for organic materials. The calcium was precipitated with ammonium oxalate, and after treatment with normal H_2SO_4 was titrated with N/100 potassium permanganate, using a micro burette. The blood was obtained from the carotid arteries under ether anesthesia.

Both bones and teeth were dissected from the tissues, thoroughly extracted with hot alcohol, measured with a vernier scale or with dividers and a steel rule and then ashed in an electric muffle furnace after drying. Some measurements were also taken of the width of metaphyses of bones from the high calcium groups after treatment essentially according to the technique used for the line test (8).

The general composition of the rations has already been referred to (1). The stock ration contained approximately, as computed from analyses, 0.60 per cent calcium, the high calcium rachitogenic ration 1.22 per cent calcium, and the low calcium ration 0.10 per cent of calcium.

A summary of representative data on our bone analyses is shown in table 1. In both young and medium sized rats on the stock ration with a pronounced decrease in growth or even loss in weight there occurred a decrease in the amount of ash expressed both absolutely and in terms of percentage of weight of bone. This decrease occurred to an even greater extent on the low calcium diet and in a shorter period of time when the weights of the animals had been affected but slightly.

Our animals on the rachitogenic high calcium diet gave the most striking results. Instead of a decrease in ash content, sodium fluoride caused an actual increase of 70 per cent in the weight of ash and 40 per cent in its percentage. In these particular instances the consumption of the rations had been equalized with all animals and the length of bone averaged the same in both. However, the weight of the bones of the fluorine fed animals averaged 18 per cent higher. The amount of bone tissue as well as ash was therefore actually increased under conditions where the effect of all food factors except sodium fluoride had been equalized. In another series

in which the food intake was not equalized, we obtained identical results. To economize space these results are not presented in the table.

It furthermore is to be noted that the addition of vitamin D to the high calcium ration maintained the same relations at a higher plane although the effect was somewhat moderated. What struck us particularly was the fact that in the fluorine fed animals not receiving vitamin D the metaphyses were linear in character while in the controls they were, as was to be ex-

TABLE 1

Effect of fluorine feeding on bone calcification

RATION (c)	NUMBER OF ANIMALS	WEIGHTS		LENGTH OF BONE		WEIGHT OF ASH		PERCENTAGE OF ASH	DURATION OF EXPERIMENT
		Initial	Final	Range	Average	Range	Average		
		grams	*grams*	*cm.*	*cm.*	*mgm.*	*mgm.*		*weeks*
Stock (a)	5	55	349	3.7–3.9	3.8	220–271	242	62.3	15
Stock + F (a)	5	60	133	2.8–3.4	3.1	84–180	123	60.3	15
Stock (a)	4	166	394	3.7–3.8	3.7	348–428	397	63.0	40
Stock + F (a)	3	172	157	3.1–3.4	3.2	183–297	242	60.5	40
High Ca (b)	9	59	88	2.1–2.3	2.3	22–28	25	25.0	6
High Ca + F (a)	10	59	84	2.1–2.3	2.3	38–49	43	36.1	6
High Ca + vitamin D (b)	10	59	81	2.4–2.7	2.6	51–67	60	45.6	6
High Ca + F + vitamin D (b)	10	59	74	2.2–2.5	2.4	50–70	61	47.4	6
Low Ca	6	61	76	2.2–2.4	2.3	32–48	39	38.9	4
Low Ca + F	12	63	58	2.0–2.3	2.1	21–40	28	36.1	4
Low Ca	9	64	79	2.3–2.5	2.4	29–46	38	37.4	6
Low Ca + F	10	65	57	2.1–2.3	2.2	21–35	28	33.5	6

(a) Analyses for these were run on tibias, all others on femurs.

(b) Animals were paired and food consumption was equalized in this entire series. Vitamin D where added was given as irradiated yeast. The basal ration was No. 2965.

(c) Fluorine when added to the ration was added as sodium fluoride to the extent of 0.15 per cent.

pected, approximately 4 mm. wide. The costochondral junctions of the fluorine fed animals were only moderately enlarged and not angulated. As this deposition of calcium occurred without stunting of growth it furnished definite visual evidence of the calcifying effect of fluorine when incorporated in high calcium diets.

In table 2 are presented data on the incisor teeth. Here it will be noted, in contrast to the results on bone, that the ash content was decreased consistently with fluorine additions, even on the high calcium diet. At

present we can see no reason for this variation in consistency from the results on bone unless it suggests itself that in the teeth of the fluorine fed animals the ratio of the weight of roots to crowns may have been greater, and as the percentage of ash in the roots of teeth is less than in the crowns, a decrease in the gross values might thus have been obtained. Unfortunately, although we measured most of the teeth on the high and low calcium diets before ashing, we did this only to determine if they had been worn down and, accordingly, we measured only the height of the crowns to the gum line. For purpose of comparison, as presented in table 2, the heights of the crowns of the upper and lower incisors have been added. In no case, will it be noted, was there more than a 14 per cent decrease in height and in one case the values were the same for crowns from the fluorine and

TABLE 2

Effect of fluorine feeding on calcification of teeth

RATION	NUMBER OF ANALYSES	RANGE IN PERCENTAGE OF ASH	AVERAGE PERCENTAGE OF ASH	AVERAGE COMBINED LENGTH OF UPPER AND LOWER INCISORS
Stock	2	76.6–76.9	76.7	
Stock + F	3	73.0–73.3	73.1	
High Ca	6	69.1–75.1	70.9	1.05
High Ca + F	5	63.4–68.6	68.0	1.05
High Ca + vitamin D	5	74.9–76.4	75.7	1.10
High Ca + F + vitamin D	7	66.2–73.9	70.6	1.05
Low Ca	2	64.5–65.1	64.8	1.25
Low Ca + F	6	56.3–61.4	57.2	1.10

non-fluorine fed animals. This makes it very improbable that the variation in ratio of crown to root was a determining factor, especially as the difference in ash in one case amounted to over 40 per cent.

We expected that blood analyses for inorganic Ca and P in the sera might give us some valuable information. On the low calcium diet the values for calcium with and without fluorine were the same, viz., 6.4 mgm. per cent; on the stock diet they were practically the same, viz., 11.9 and 11.7, and on the high calcium diet possibly slightly different, viz., 12.2 and 11.5 respectively. The phosphorus values were 7.8 and 8.8 with and without fluorine on the low calcium diet and 4.3 and 2.9 respectively on the high calcium diet. No phosphorus determinations were run on sera from the stock diet. These values are essentially what might be expected from the composition of the diets irrespective of fluorine influence except on the high calcium rachitogenic diet where the calcium and phosphorus were apparently increased.

It appears, therefore, that the problem merits further study. However, so far as our results reveal in this paper and in the preceding one (1), it is evident that the Ca content of the diet is an important factor in determining the effect of fluorine on body growth, maintenance and the ash content of bone and teeth.

SUMMARY

The addition of 0.15 per cent sodium fluoride to the diet of young rats produced a variable effect on ash content of bone. On a diet low in calcium and on a stock diet of moderate calcium content the ash content was decreased absolutely and percentagely. On a high calcium rachitogenic diet it was definitely increased. However, the total ash in the incisor teeth was decreased in all cases. It was found impossible to correlate these results with any consistent change in the amount of inorganic Ca and P in the blood sera.

BIBLIOGRAPHY

(1) Hauck, H. M., H. Steenbock and H. T. Parsons. This Journal, 1933, ciii.
(2) Forbes, E. B., J. O. Halverson, J. A. Schultz et al. Ohio Agric. Expt. Sta. Bull., 347, 1921.
(3) Tolle, C. and L. A. Maynard. Proc. Amer. Soc. of Animal Prod., 15, 1928, 15.
(4) McClure, F. J. and H. H. Mitchell. Journ. Biol. Chem., 1931, xc, 297.
(5) Chaneles, J. Rev. Soc. Argent. de Biol., 1929, v, 336.
(6) Fiske, C. H. and Y. Subbarow. Journ. Biol. Chem., 1925, lxvi, 375.
(7) McCrudden, F. H. Journ. Biol. Chem., 1911–1912, x, 187.
(8) McCollum, E. V., N. Simmonds, P. G. Shipley and E. A. Park. Journ. Biol. Chem., 1922, li, 41.

ADRENALECTOMY IN THE RAT

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Until a few years ago it was generally assumed that a large percentage of rats survived bilateral adrenalectomy for a period of several weeks or indefinitely. The evidence indicated that the presence of accessory cortical tissue made this indefinite survival possible.

Among those workers stating that large percentages of rats (generally 50 per cent or more) survived after adrenalectomy were: Philipeaux (1856), Harley (1858), Boinet (1895a), Lewis (1923), Scott (1925) Rogoff and DeNecker (1925), Jaffe (1926), Torino and Lewis (1927), Wyman (1928a), and Wyman (1928b).

The conclusions reached by these above workers was until recently widely accepted, although they had earlier been challenged by Strehl and Weiss (1901) and by Christiani and Christiani (1902), who stated that death was the invariable consequence of adrenalectomy in the rat.

The agreement between modern workers on this subject was recently interrupted, however, by Pencharz, Olmsted and Giragossintz (1930), (1931), who announced that out of 62 rats adrenalectomized by them none survived more than 20 days. No definite explanation was given of this unusual result, although it was inferred that perhaps the great care taken to remove all of the gland "with the bit of fat immediately surrounding it" was responsible for their findings.

Freed, Brownfield, and Evans (1931) stated definitely that at least in male rats adrenalectomy was always fatal if one-quarter inch of the pedicle of the gland was removed along with the adrenals proper. If this precaution were not taken 25 per cent of the animals were unaffected by the operation. Kutz (1931), using a series of 57 28-day-old rats, reported that 56 were dead by the tenth day after adrenalectomy.

Martin (1932) reported a survival of 20 per cent of female and 25 per cent of male adrenalectomized rats in over 200 cases. He has since informed the author in private communication that extended studies on his colony shows that less than 5 per cent survive adrenalectomy. In all cases he removed the surrounding fat, connective tissue and pedicle in the manner of Freed et al.

These papers appearing since 1930, whether or not they reported an invariable fatality, did indicate that rats die after adrenalectomy in much greater numbers than most previous workers had found. Nevertheless, while these results were being obtained, workers in other laboratories had, as usual, been getting a large percentage of survivals. Marmoston-Gottesman and Perla (1932) in a recent publication state, "The mortality from suprarenalectomy alone in rats during the first three weeks is less than 10 per cent in our laboratory." Thus, it is seen that a dilemma presents itself. While workers in one laboratory obtained a 10 per cent mortality in the first three weeks, investigators elsewhere obtained 100 per cent mortality in that same length of time. Such directly opposed results cannot be explained on the basis of variable, individual interpretation. The criterion is simply whether or not a rat is living or dead!

A search of the literature fails to provide information for a correlation of this variety of findings. The papers of Pencharz et al. and Freed et al. suggest that the type of operation may markedly influence the results. Several workers have intimated that careless operative technique, resulting in fragments of the main gland being left behind, have accounted for some of the high survival figures. This may be true and it is certain that unless the adrenals are removed intact and uncrushed there is no way of telling whether or not the extirpation is total. But adrenalectomy is a relatively simple operation in the rat and can be done neatly by any investigator of ordinary competence after a little practice, so it is altogether improbable that bad surgery could have accounted for the high survival figures reported by many experienced workers.

This study was undertaken to find, if possible, some of the causes of such diversity of reports. Specifically, an attempt was made to determine if age, sex, strain of animals or the excision of extra tissue around the adrenals could be factors influencing the life-span. Further observations were made and are here reported on the adrenal insufficiency syndrome in the rat.

A preliminary report of this work has been published (Gaunt, 1932). It is here extended and reported in full.

EXPERIMENTAL METHODS AND MATERIALS. Animals were obtained from five different colonies: one of these was a strain of hooded rats long bred at the State University of Iowa and later at New York University by Dr. H. O. Haterius; another was from an albino colony bred by Doctor Haterius; another was from an albino strain reared for several years in the psychological laboratory of Princeton University, coming originally from the Wistar colony; another strain came from a dealer in Baltimore and was of unknown origin; the fifth colony which will be subsequently referred to as the "T-Colony" came from an animal fancier in Trenton, N. J. These latter animals, although not reared under laboratory conditions, were

obviously healthy and well nourished. They were reputed by their owner to have been the descendants of an original mating between two albinos of South American stock, reared for pet purposes. Animals from part of these colonies were mated in this laboratory and the offspring used.

The results of 185 bilaterally adrenalectomized rats of both sexes ranging in age from 35 days to 10 months are here reported. Care was taken to select no animal that was not to all appearances in perfect condition of health. And no animal was recorded as having died of adrenal insufficiency if any known complicating factor could have interfered with the diagnosis. Quite a number were discarded because at autopsy severely congested lungs were found, but doubtless this trouble was, in most cases, the consequence of adrenal insufficiency rather than the cause of death. To demonstrate that the operated rats were really dying of adrenal insufficiency a series from all colonies were revived from their terminal prostration with the cortical hormone, kindly supplied by Drs. W. W. Swingle and J. J. Pfiffner.

Operative technique and post-operative care. The dorso-lumbar approach was used, separate incisions through the skin and muscle being made for each gland. If the incision was properly placed the gland could be directly approached and quickly removed. The approach on the right side was high in the angle between the last rib and the vertebral column. On the left side a slightly more posterior and central approach is preferable. A pinch with a pair of forceps on the blood supply of the gland causes complete hemostasis. In approximately half of the cases of all colonies an attempt was made to take out with the gland the surrounding fat and as much as possible of the pedicle of the gland, because according to Freed, Brownfield and Evans by this means only can uniform mortality be expected. In the rest a careful attempt was made to remove only the adrenal and its capsule leaving intact and uninjured, as far as possible, all

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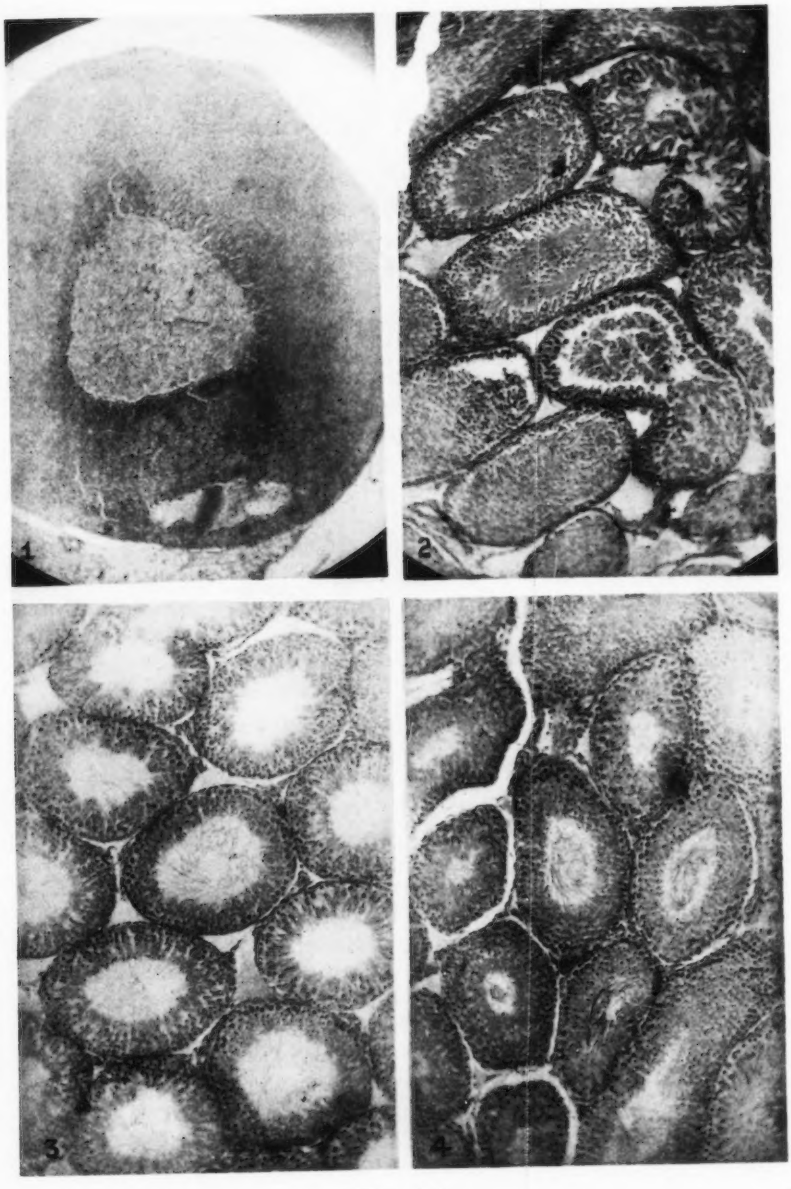
Fig. 1. Accessory adrenal with well-defined accessory medulla. This accessory gland was found anterior and medial to the site of the excised left adrenal. The animal was autopsied at 3 months after operation, and had been in excellent condition until that time (rat H-75).

Fig. 2. Shows a section through the testis of an adult rat which was killed for autopsy near the point of death 18½ days after adrenalectomy. The tubules pictured here show considerable disorganization. This region was selected to photograph because it showed the maximum degree of degeneration that could be found in any of the cases studied. Only a few tubules in the testis were thus disturbed. The rest were normal (rat T-169).

Fig. 3. Shows section through testis of adult rat that lived 14 days after adrenalectomy and was killed when in the terminal stages of adrenal insufficiency. It is apparently normal and is the characteristic finding in animals dying of adrenal insufficiency (rat H-147).

Fig. 4. Shows section through testis of normal control adult rat.



Figs. 1–4

surrounding tissue. The distinction between these two types of operation is definite, as considerable tissue proximal to the gland can be excised if it is desired. The muscle layers were closed by a single silk suture and the skin incision, one of about 1 cm. in length, was likewise closed by a single suture. Aseptic precautions, including boiling of instruments, sterilizing cutting edges with alcohol, surrounding operation site with sterile cloths, washing hands in alcohol, painting skin of animal with iodine or with mercurochrome and alcohol, were used.

In a few of the first cases one gland only was removed at a time, but this made no difference in the life-span and is in the rat, as many workers have concluded, a waste of time and effort.

Sodium amytal was generally used as an anesthetic because with it a quick, aseptic operation is easier to perform when an assistant to give ether is not available. The long anesthetic period which makes sodium amytal an unfavorable anesthetic for ordinary use in cats is not seen in rats. Rats generally recover within two hours or less. The dosage, 0.001 cc. of 10 per cent solution per gram of rat, was recommended by Dr. J. S. Nicholas of Yale University (private communication) and found satisfactory. In case this dose does not produce a total anesthesia a small amount of ether administered while the animal is being prepared for operation will insure a deep anesthesia, making further attention unnecessary. Males generally require more amytal than females and Nicholas uses just twice the above dose for males, but we have not found necessary such a marked dosage difference for the sexes. One precaution is necessary: while recovering from amytal rats should be kept in a warm place (preferably warmer than an animal room at 80°F.). Otherwise a severe temperature loss may ensue. Ether anesthesia alone was used when an anesthetist was available.

The animals were fed on a standard diet containing all necessary food factors. The diet is important in survival as Scott (1923) has pointed out. When rats from other laboratories were used they were kept for several days after arrival before operation in order that they might become accustomed to the diet and recover from any possible ill effects of transportation. Operated rats were kept singly or in pairs in cages placed in a room evenly heated at 80°F. A warm, evenly-heated room is imperative if maximum survival is desired. If, accidentally, a marked variation in room temperature, either up or down, occurs a large number of sudden deaths among operated animals is the invariable consequence; and these cases must be discarded as far as survival figures are concerned. Daily weight records, and in many cases, daily temperature records were kept for at least three weeks after operation. Post-mortems were always performed and a search made for accessory tissue.

SURVIVAL RESULTS. *a. Colony differences:* The results of these experi-

ments indicate that different colonies of rats differ remarkably in their post-adrenalectomy survival. Such, indeed, is the extent of this difference that it could explain, in large part, the previous discordant reports in the literature. Animals from all colonies were classified separately, but the records of four of the five groups were found to be almost identical and hence are considered here together (fig. 5).

These four colonies gave results essentially similar to those reported by Pencharz, Olmsted and Giragossintz. There was an almost invariable mortality by or before the fifteenth day, and at 20.5 days 95 per cent of the animals were dead. Five per cent lived a month or longer. Those

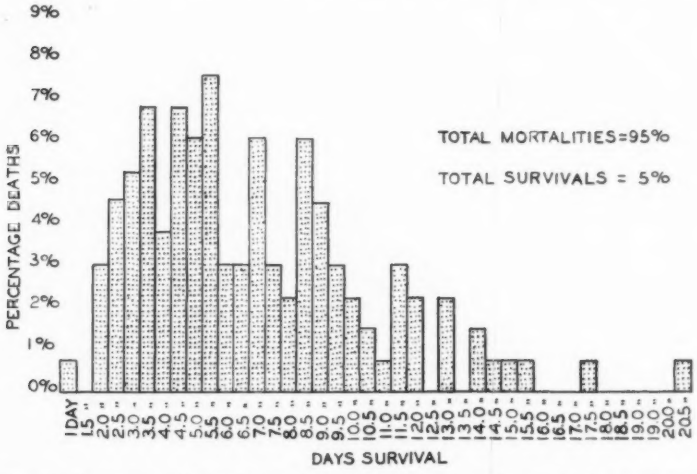


Fig. 5. Shows the life-span distribution of 130 adrenalectomized rats from four colonies in which there was a 95 per cent mortality. The abscissae represent days survival in one-half day intervals after adrenalectomy. The ordinates represent the percentage mortality. The 5 per cent "survivals" means that percentage of animals that lived for 30 days or longer.

animals living a month or longer are arbitrarily referred to here as "indefinite survivals," because most of them live for long periods—at least for two or three months at which time they were generally killed for autopsy. In these colonies one rat, surviving more than a month, died at 32 days, while the rest were apparently in good condition at autopsy.

Swingle and Pfiffner (in press) find a 9.8 day average survival for untreated adrenalectomized cats; and Stewart and Rogoff's (1928) best series of survivals of adrenalectomized dogs averaged 9.9 days. These survival figures for cats and dogs compare fairly closely with the survival results of our four colonies of rats. Probably, then, such a life-span is that

to be expected in any adrenalectomized mammal relatively free of functional accessory cortical tissue.

The fifth or "T-Colony" of rats showed an entirely different picture (fig. 6). Fifty-five animals from that colony have been operated. Of that number 30, or 55 per cent, died within one month after adrenalectomy. Eight lived longer than one month but died at 32, 35, 42, 51, 53, 60, 62, and 64 days. The other 13 were apparently in excellent condition, showing no signs of chronic adrenal insufficiency, at the time of autopsy from 2 to 3 months after operation. The 30 which died within 30 days showed an average life-span of 14.4 days, just twice that of the other four

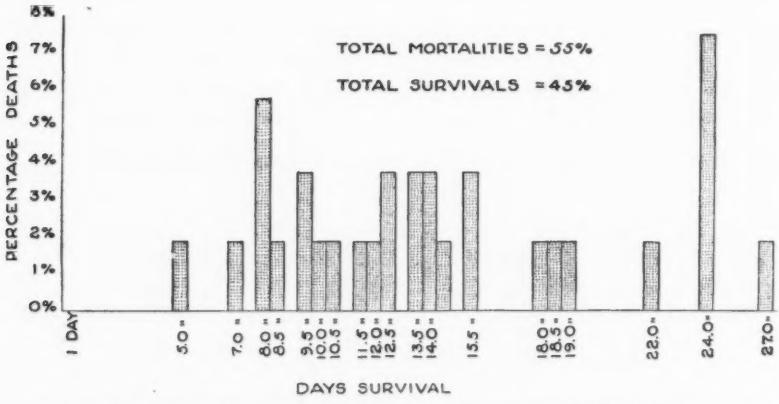


Fig. 6. Shows the life-span distribution of 55 adrenalectomized rats from the "T-Colony" in which there was a 55 per cent mortality. The scale is the same as that of figure 1, and it is seen that these animals showed a longer and more varied life-span with a survival for a month or longer of 45 per cent. The abscissae represent days survival after adrenalectomy in one-half day intervals. The ordinates represent the percentage of mortalities occurring at any given day.

colonies. The deaths occurred between 5 and 27 days. It is noteworthy that in this colony no deaths occurred before the fifth day, a result very unlike that found in the other colonies. The findings with this colony compare very favorably with many of the earlier reports in the literature.

It should be emphasized that exactly the same operative and post-operative technique was employed for the animals of all colonies. Much of the work on two of the colonies in which there was a high and rapid mortality has been done since the operations on the "T-Colony." And when it was seen that such marked differences were occurring additional animals from the first two colonies investigated were adrenalectomized to see if any unsuspected differences in technique or care could have accounted

for the inconsistent results. None were found. Later animals from the different colonies were raised side by side in this laboratory in the same room and on the same diet. When later adrenalectomized the same survival differences were noted.

To check the effects of surgical trauma a series of operations was done in which one gland was removed entirely and all but a fragment of the second was removed. In no case did death result; and, although this operation might temporarily slow up the growth rate, no weight loss comparable to that in adrenal insufficiency was recorded. Oftentimes this control operation occasioned no weight loss. An abbreviated protocol from this series follows:

Rat H-146. Age 2.3 months. Weight 132 grams. Female. On 2/5/32 left adrenal removed intact and small piece of the right purposely left behind. Twenty-four hours after operation animal weighed 122 grams. Forty-eight hours after operation weighed 125 grams. Thereafter the weight increment was steady and rapid.

b. Survival difference due to sex. Sex made little difference in the average life-span of any colony, the average for males surviving less than one month from all five colonies being 8.5 days and for females 7.7 days. Eighteen and two-tenths per cent of the females from all colonies survived 30 days or longer, but only 10.4 per cent of the males. This small percentage difference between males and females, in view of the small numbers involved, is doubtless without significance. Wiesel (1898) reported that 50 per cent of male rats have accessory cortical bodies between the lower pole of the epididymis and the testis, and that this tissue hypertrophies after adrenalectomy. Some workers have advanced this as an explanation of the observation that the life-span of males is higher than that of females. Martin, for instance, noted that 5 per cent more males than females survived indefinitely. It is improbable, however, that any significant survival difference due to sex exists. For further observations on this subject see Marti (1923).

c. Survival difference due to age. Rats between 35 days and 2 months of age were slightly more susceptible to the operation than older ones, their average life-span in the four colonies represented in figure 1 being 5.7 days, compared to the 7 days average for all animals of these four colonies classified together. These results are similar to those given by Freed, Brownfield and Evans, who state, "The immature males died regularly [after adrenalectomy] in 4 to 6 days. Mature males in a smaller series lived on the average a day or two longer." All of the indefinite survivals in these four colonies were animals over two months of age. Rats of the "T-Colony" are not included in these calculations of age differences because their exact ages were unknown in the animals not raised in our laboratory. There was, however, little apparent age difference.

d. Survival difference due to removing tissue surrounding adrenal. An attempt was made in about half of the animals of every colony to do the operation of Freed et al., Pencharz et al., and Martin, in which the tissue surrounding the adrenal was removed along with the adrenal proper. These authors are of the opinion that such an operation either insures 100 per cent mortality or at least reduces the percentage of survivals. We have found no difference, however, of any probable significance between this operation and one in which care is taken to remove the adrenal only. It did not influence the average life-span in any colony. The only evidence obtained indicating that the removal of extra tissue modifies the results was that in all of the 5 per cent surviving indefinitely in the four colonies represented in figure 1 the adrenals only had been removed. In the T-Colony 26 animals were carefully operated to check this point. Seven of the animals that died within a 30 day period had extra tissue taken with the gland and 6 did not. Of the 13 survivals 6 had the surrounding tissue removed and in 7 the adrenals only were excised. In this colony, clearly, the type of operation had no effect on the results.

It is of interest in this connection that long ago Boinet (1895b) tried without effect a similar type of experiment. He used an electrocautery to destroy any accessory adrenals adjacent to the main glands. He cauterized the tissue around the kidney and the renal blood supply that cannot be removed surgically. Of the 12 rats, however, thus treated, 7 survived indefinitely.

We have made, in this connection, a histological study of all the tissue removed in several of the cases where extra tissue was taken along with the adrenals. This has failed to reveal any microscopic rests of accessory cortical tissue which might, if left intact, be expected to hypertrophy and influence the life-span.

5. Symptoms of adrenal insufficiency and reaction to cortical hormone. a. General symptoms. The rat demonstrates classical symptoms of adrenal insufficiency, characterized by: profound asthenia, loss of body weight, loss of body temperature and severe gastro-intestinal lesions. In varying percentages of the cases one sees, hypertrophy of thymus with congestion or small hemorrhagic spots, congestion of lungs, liver, spleen, pancreas, etc. Total anorexia generally occurs as only a terminal symptom, and the rat, unlike a cat or dog, oftentimes will eat lettuce or some food of which it is particularly fond until a few hours before death, so that in many cases food is found in the stomach at autopsy. Diminished food intake, however, is an invariable symptom. The incidence of diarrhea as a symptom has been observed in our animals in only a very few cases and in much less proportion than some have reported. It is doubtful that diarrhea can be included in the adrenal insufficiency symptomatology. An interpretation of the common occurrence of lung congestion at autopsy is difficult. In

over half of the cases it was not seen and in others it occurred generally with no other evidence of respiratory infections. The well-known susceptibility of adrenalectomized mammals to respiratory infection, however, casts doubt as to whether or not adrenal insufficiency alone caused death when an adrenalectomized rat shows severe lung complications at autopsy. Animals showing such a severe congestion were not included in these totals. But the probability is that this exclusion was not justified, because a comparison of the average life-span of rats showing at death, 1, no lung congestion; 2, slight lung congestion, and 3, severe lung congestion, shows for the first two categories an almost identical life-span average and for the latter group an average of about one day less.

b. Heat regulation. In all mammals a loss of body temperature seldom fails to accompany the acute symptoms of adrenal insufficiency. The rat presents this symptom in a more striking manner than either the cat or dog. For adult rats it is almost a rule that the rectal temperature will drop below 90°F. a few hours before death; and we have recorded many cases of rectal temperatures between 80° and 85°F. from two to six hours before death. It may be depended upon that when a rectal temperature of 90°F. is reached death is imminent and will occur within less than 12 hours. When adult animals were being revived from their comatose terminal condition with the cortical hormone, injections of the extract were seldom made until the rectal temperature fell to 90°F. or below. That temperature furnishes a rough but not invariable criterion of the critical stage after which revival cannot be depended upon. Young animals seem to develop the terminal symptoms of adrenal insufficiency more rapidly than adults and if certain revival with the cortical hormone is desired injection should not be delayed after the rectal temperature has dropped to around 94°F., if the animal is at that time prostrate. Body temperature is the best objective criterion we have found of the actual condition of rats in the final stages of adrenal insufficiency but it is not entirely dependable.

A drop of rectal temperature does not occur as an early symptom of adrenal insufficiency. Even though the animal is progressively losing body weight the temperature will remain normal. Normal temperature in the rat fluctuates between 98° and 101°, according to the individual and the time of day. The first temperature drop due to adrenal insufficiency is a slight one generally to approximately 97°F., which may persist for a few hours to two or three days; and then as the symptoms become acute it falls precipitously. Wyman and tum Suden (1929), and Hartman, Brownell, and Crosby (1931) have studied the reaction of adrenalectomized rats to cold and found them much more susceptible than normal animals. This fact makes it imperative that the animals be kept in a warm, evenly-heated room.

Normal rectal temperature is regained completely after treatment with the cortical hormone. From 30 to 60 minutes after intraperitoneal injection—when the injection is given when the rat's temperature is approximately 90°F.—the temperature continues to drop, then it begins to climb and regains a normal range in 5 or 6 hours. In much less time the animal will have regained activity and appetite. The following protocol gives an idea of the fall of temperature and weight in an adrenalectomized rat:

Rat H-120. Male. Age 2.5 months. Weight 150 grams.
1/17/32 a.m.—Double adrenalectomized.
1/18/32 p.m.—Weight 148 grams; rectal temp. 99°F. Condition good
1/19/32 p.m.—Weight 146 grams; rectal temp. 99°F. Condition good
1/20/32 p.m.—Weight 145 grams; rectal temp. 98°F. Condition good
1/21/32 p.m.—Weight 145 grams; rectal temp. 98°F. Condition fair
1/22/32 p.m.—Weight 140 grams; rectal temp. 98.5°F. Condition fair
1/23/32 p.m.—Weight 137 grams; rectal temp. 97°F. Weak and inactive
1/24/32 p.m.—Weight 130 grams; rectal temp. 96°F. Marked symptoms.
1/25/32, 9:30 a.m.—Rect. temp. 94°F.; looks very weak. 1:15 p.m.—Rectal temp., 89.5°F.; animal can hardly move. 9:30 p.m.—Rect. temp. 88°F.; animal prostrate. Found dead next morning.

The next protocol shows the weight and temperature reaction of a young rat that came down with adrenal insufficiency, was revived, and then allowed to die after only two injections in rapid succession of the adrenal cortical hormone.

Rat PP-190. Male. Age, 41 days. Weight 93 grams. Rect. temp. 99°F.
3/20/32 a.m.—Double adrenalectomized.
3/21/32 p.m.—Wgt. 94 g.; Rectal Temp. 99°F. Condition good
3/22/32 p.m.—Wgt. 89 g.; Rectal Temp. 99.5°F. Condition good
3/23/32 p.m.—Wgt. 85 g.; Rectal Temp. 99°F. Condition good
3/24/32 p.m.—Wgt. 86 g.; Rectal Temp. 98°F. Condition good
3/25/32 p.m.—Wgt. 86 g.; Rectal Temp. 98.5°F. Condition good
3/26/32 p.m.—Wgt. 87 g.; Rectal Temp. 99°F. Condition good
3/27/32 p.m.—Wgt. 85 g.; Rectal Temp. 98°F. Quiet and Inactive
3/28/32 p.m.—Wgt. 86 g.; Rectal Temp. 97°F. Slightly weak and lethargic
3/29/32, 5:45 p.m.—Weight 85 grams; Rect. temp., 96°F.; obviously coming down with adrenal insufficiency; convulsive twitching of muscles; asthenia. 7:40 p.m.—Rect. Temp. 94°F.; animal prostate and apparently near death; given 1 cc. cortical extract intraperitoneally. 9:50 p.m.—Rect. temp. 95°F.; animal much better and can walk; given another dose of 1 cc. cortical extract subcutaneously. 11:00 p.m.—Rect. temp. 96.5°F.; animal eating and fairly active. Extract discontinued.
3/30/32, 9:30 a.m.—Rect. temp., 98°F.; animal active; looks normal. Maintained normal temperature and activity for rest of day. Weight 85 grams.
3/31/32, 9:30 a.m.—Rect. temp., 96°F.; coming down again with adrenal insufficiency. Gradually got worse during day. 10:30 p.m.—Rect. temp., 93°F.; weight 80 grams.
4/1/32.—Found dead a.m.

The above case is typical of two characteristics of young adrenalectomized rats: the temperature in the terminal stages is, compared to adults, high; and there is no marked drop in weight during terminal stages.

c. Body weight. For mature animals body weight is the most reliable check on the early progress of adrenal insufficiency. While rats are to all appearances normal in other respects after adrenalectomy a progressive loss of weight indicates an eventual mortality (see previous protocol, rat H-120).

Fifteen to 20 per cent of the body weight is generally lost before death. Pencharz et al. stated that in their animals there was not a single case that showed a gain of weight after adrenalectomy. This in our experience has not been an invariable rule, but it is generally true for mature animals.

In young animals there may be a gain of weight for the first few days after operation—a fact that speaks against any severe effects of surgical trauma—and then as adrenal insufficiency becomes more acute a slight weight loss will occur before death. This is most often seen in those cases of extended survival, as indicated in the following:

Rat T-175. Young female, weighing 75 grams, gained to a weight of 108 grams at 17 days after adrenalectomy. She then lost weight until at death 32 days after operation her weight was 95 grams.

Young as well as mature animals sometimes show a progressive loss of body weight, however; but as Jaffe has pointed out, the weight loss in adrenalectomized rats is largely at the expense of body fat, so such a high percentage loss is never seen in young as in mature animals. (See previous protocol, rat Pp-190.)

6. Accessory cortical tissue in relation to life-span. Accessory cortical tissue in rats is in many cases difficult to locate. In our animals which survived for as long as two months after operation it was found in macroscopic amounts in approximately one-third of the cases, and always on the left side, not far from the site of the excised adrenal proper. That these were true accessories and not fragments of the main gland left behind at operation was checked by examination of the excised gland which was preserved at the time of removal. No case of indefinite survival was regarded as significant if the glands were accidentally crushed at the time of removal. In the animals surviving indefinitely, in which no macroscopic cortical tissue could be found, a microscopic study of the renal vein and the tissue immediately surrounding it made by Mr. Maurice Laufer, revealed accessories in more than half of the cases. There is every reason to assume that accessory tissue was present in cases of extended survival even though it could not be demonstrated. In part of the indefinitely-surviving cases the microscopic accessory cortical tissue found was in very small amounts and probably not of sufficient size to maintain life. Doubtless other undiscovered rests were present.

Abelous and Langlois (1895) said that in 10 out of 11 rats examined, microscopic accessories could be found in the neighborhood of the renal veins. And Wiesel (1898) reported its presence in 50 per cent of male rats around the epididymis. Many workers have reported gross accessory tissue in rats living for long periods after adrenalectomy, but it is rarely found in macroscopic amounts prior to adrenalectomy. Pencharz et al. have reported one case out of 500 in which a macroscopic accessory was seen either before or after adrenalectomy. It is supposed that a hypertrophy of microscopic cortical rests brings them into evidence after adrenalectomy, in the cases where they are seen.

An assumption of the presence of accessory glands, even though they are not located, is necessary to explain the life-span in those rats in which adrenalectomy is eventually fatal but the life-span long. When rats are kept alive and in good condition on the hormone of the adrenal cortex and then taken off the extract, the survival period ranges from 3 to 10 days. Rats in acute adrenal insufficiency are more quickly revived than either dogs or cats. This shows that the rat is very susceptible to the presence of the cortical secretion and can live for only a short time in its absence. Therefore, it seems unnecessary to discard, as some have done, all cases dying within less than five days after adrenalectomy as being due to post-operative complications. If adrenalectomy is actually total a rat may well, though not necessarily, be expected to die after three days of adrenal insufficiency. Animals dying within three days can be revived at the point of death with the cortical hormone and restored to good health. If rats live then for any considerable period of time after operation it must be due to accessory cortical tissue somewhere in the body. That surgical trauma need not be involved in early deaths can be seen from the quick recovery of the animals after operation and the fact that control operations of equal severity are of very slight, if any, deleterious effect. In our "T-Colony," a group which in all probability possessed considerable accessory tissue, the earliest death was at five days. But in the other colonies, which obviously did not have enough accessory cortical tissue to maintain life for long periods of time, there were large numbers of mortalities within less than five days. Such a variable result can best be explained by presuming the presence in some of more accessory adrenals than in others.

We have had three cases in which rats came down after adrenalectomy with unmistakable crises of adrenal insufficiency, were revived with cortical extract, and then survived indefinitely after only a few extract treatments. Apparently the treatment had maintained life long enough for cortical accessories to develop after which further administration of the hormone was not essential. Macroscopic accessories were later located in these animals.

Accessory adrenal medullary tissue is rarely reported in the rat. We

have found two cases, however, where it was present. Both were inside and completely surrounded by an encapsulated cortical accessory, forming a perfect miniature adrenal (plate 1, fig. 1). Both were on the left side. One was macroscopic and placed a little anterior and medial to the site of the adrenal proper; the other was detected only after histological study of the tissue around the left renal vein. Both animals had been killed for autopsy after having survived adrenalectomy for more than two months.

7. *Lactation and reproduction.* A systematic study of the reproductive functions of adrenalectomized rats was not undertaken. Treatments of that subject have been given by Novak (1913), Wyman (1928b), Martin and others. A few observations, however, were of interest. Twelve of the adrenalectomized females gave birth to litters of young. Part of them had been impregnated at the time of operation and part afterwards. The litters ranged in numbers from 2 to 10 and were apparently normal in every respect. In only two cases have the litters been adequately raised, even though, in part of the cases, the mothers were apparently in good condition, and lived for several weeks after the weaning time of the pups. The young have either been greatly stunted in their growth, as in one case; or, as in nine cases, have starved to death within 3 to 13 days apparently because of insufficient lactation on the part of the mother. In some cases the mother would make no effort to care for her young; in others they attempted to care for them normally. The two animals which raised their young normally showed macroscopic accessories; the others did not.

The mothers in these cases were on the same diet as that used for normal animals which were reproducing and lactating normally. This observation is, probably, only another manifestation of chronic adrenal insufficiency (in the sense of Jaffe), which renders an animal incapable of meeting an excess, sustained, metabolic demand. It could possibly be due to an inadequate production or release of the lactation hormone of the pituitary, as Martin has apparantly shown that such a deficiency of the gonad-stimulating factors of the pituitary exists in animals suffering from adrenal insufficiency. Such effects resemble those caused by vitamin E deficiency. This could not have been the cause, however, as the diet was rich in vitamins and lettuce in abundance was supplied from 3 to 6 times per week.

It is possible that the lactation failure reported by Carr (1931) in his adrenalectomized, extract-treated rats was due to an inadequate unit dosage, resulting in a chronic adrenal insufficiency. Adrenalectomized dogs kept alive in the laboratories of Prof. W. W. Swingle on cortical extract reproduce and lactate normally (in press).

8. *Effects of adrenalectomy of the testis.* A supposed relationship between the adrenal cortex and the gonads has commanded the attention of a number of workers during past years and is being studied in several laboratories at the present time. Of interest in this connection is the effect of adrenal

insufficiency on the testis. Reports in the literature indicate that the rat alone, among mammals studied, shows a pathological condition of the tubules of the testis as a result of fatal adrenal insufficiency. Jaffe (1923), working on rabbits, found tubular degeneration rare after adrenalectomy and concluded that it was due, when seen at all, merely to the general poor condition of the animal. McMahon and Zwemer (1929) studied the testes of adrenalectomized cats and reported some changes in the interstitial tissue but none in the tubules.

Novak studied the development of the entire genital system of the rat after adrenal ablation. He found a hypoplasia of the genitalia including the testis. There was a cessation or diminution of spermatogenesis and all of the effects were more marked the younger the animal. The results, he stated, were unlike those resulting from nutritional disturbances.

Recently Freed, Brownfield, and Evans investigated the tubules of the testes of rats dying of adrenal insufficiency and reported very marked degeneration, as severe in mature as immature animals. These authors state, "histologically, the spermatic tubules were ragged, fragmented, and disorganized; clumps of cells lay in the lumina and often only the germinative layer remained in position. In the adult testes the spermatocytes were severely affected, staining ghost-like with eosin."

We have studied the testes of 20 adrenalectomized rats which were from 2 to 10 months old. The animals lived from 75 hours to 27 days after operation. The testes were fixed in Bouin's fluid either a few minutes after death, or, as in most cases, the animals were killed when in the final stages of adrenal insufficiency and the testes fixed immediately. Preparations were stained either in Delafield's hematoxylin and eosin or in Heidenhain's iron-alum-hematoxylin, and cleared either in xylol or cedar oil. Our material failed to show the picture described by Freed, Brownfield and Evans (plate 1, figs. 3, 4). In most of them an active spermatogenesis was proceeding and both the cytological and histological detail was indistinguishable from normal.

Rarely, tubules were seen in a few animals that show some tubular disorganization is under way (plate 1, fig. 2); and the description of them might be roughly that given by Freed et al. Such changes are generally manifested in a cellular disarrangement rather than in any cytological abnormality. Pyknotic nuclei are very rarely, if ever, present. If seen at all these changes are always in a small percentage of tubules, and, as stated above, in only a small number of animals.

Such changes as may occur, if they are really pathological at all, are too rare and slight to constitute evidence of any adreno-gonadal relationship. The severe physiological upsets that constitute the adrenal insufficiency syndrome might well entail an effect on the testis that would not be specifically and primarily due to a lack of the adrenal cortical hormone.

The author takes pleasure in acknowledging his indebtedness to Prof. W. W. Swingle for suggesting this problem and for much aid, generously given, in carrying it to completion. Acknowledgments are made to Dr. H. O. Haterius of New York University and to the Psychological Laboratory of Princeton University for kindly furnishing many of the animals used in this work. Mr. Maurice Laufer made histological sections of some of the accessory cortical tissue found in these animals.

SUMMARY AND CONCLUSIONS

1. The survival of 185 bilaterally adrenalectomized rats from five colonies was studied and a marked survival difference noticed in the animals from one colony.

2. Four of the colonies gave practically identical results, a 95 per cent mortality within 20.5 days, with an average life-span of seven days.

3. Animals from a fifth colony, on the other hand, showed a 50 per cent survival for 30 days or longer after adrenalectomy, with an average life-span of 14.4 days for those that died. The extent of these colony differences is sufficient to explain many previous diverse reports in the literature.

4. The average life-span of animals under two months of age from the four colonies in which there was a high mortality was 5.7 days, or 1.3 days less than the average for all of these animals classified together.

5. Sex made no consistent difference in the life-span. The average life-span of females was one day less than that of males, but a higher percentage of females than of males survived indefinitely.

6. Previous reports that adrenalectomy in the rat is uniformly fatal only when certain tissues around the adrenal proper are removed along with the gland were not verified.

7. Accessory cortical tissue, either in macro- or microscopic amounts, was generally found in those animals that lived over one month.

8. Adrenalectomized female rats, capable of bearing normal litters, rarely lactated in amounts adequate to raise their young in normal fashion if gross accessory cortical bodies were not present.

9. No changes of probable consequence were observed in the tubules of the testes of rats dying of adrenal insufficiency.

BIBLIOGRAPHY

Abelous and Langlois. 1895. Compt. Rend. Soc. Biol., ii, 334.

Boinet, E. 1895a. Compt. Rend. Soc. Biol., ii, 162.

1895b. Compt. Rend. Soc. Biol., ii, 498.

Britton, S. W. 1931. This Journal, xcix, 9.

Carr, J. L. 1931. Proc. Soc. Exp. Biol. and Med. xxix, 131.

Christiani, H. and A. Christiani. 1902. Journ. de Physiol. et de Path. Génér., iv, 837.

Freed, S. C., B. Brownfield and H. M. Evans. 1931. Proc. Soc. Exp. Biol. and Med., xxix, 1.

GAUNT, R. Proc. Soc. Exp. Biol. and Med., xxix, 523.
HARROP, G. A., J. J. PFIFFNER, A. WEINSTEIN AND W. W. SWINGLE. 1932. Proc. Soc. Exp. Biol. and Med., xxix, 449.
HARLEY, G. 1858. Trans. Path. Soc. Lond., ix, 40: (from BIEDL).
HARTMAN, F. A., K. A. BROWNELL AND A. A. CROSBY. 1931. This Journal, xcviii, 674.
JAFFE, H. L. 1923. Journ. Exp. Med., xxxviii, 107.
1926. This Journal, lxxviii, 453.
KUTZ, R. L. 1931. Proc. Soc. Exp. Biol. and Med., xxix, 91.
LEWIS, J. T. 1923. This Journal, lxiv, 503.
MARMORSTON-GOTTESMAN, J. AND D. PERLA. 1932. Journ. Exp. Med., lv, 109.
MARTIN, S. J. 1932. This Journal, c, 180.
NOVAK, J. 1913. Arch. f. Gynk., ci, 36.
MCMAHON, H. E. AND R. L. ZWEMER. 1929. Amer. Journ. Path., v, 491.
PENCHARZ, R. I., J. M. D. OLMSTED AND G. GIRAGOSSINTZ. 1930. Sci., lxxii, 175.
1931. Physiol. Zool., iv, 501.
PHILIPEAUX. 1856. Compt. Rend. Acad. Sci., xliii, 904.
ROGOFF, J. M. AND DENECKER. 1925. Journ. Pharm. Exper. Therap., xxvi, 243.
ROGOFF, J. M. AND G. N. STEWART. 1928. This Journal, lxxxiv, 660.
SCOTT, W. J. M. 1923. Journ. Exper. Med., xxxviii, 543.
STREHL, H. AND O. WEISS. 1901. Pflüger's. Arch., lxxxvi, 107.
SWINGLE, W. W. AND J. J. PFIFFNER. 1931. This Journ., xvci, 153.
TORINO AND J. T. LEWIS. 1927. This Journal, lxxxi, 405.
WIESEL, J. 1898. Centralbl. f. Physiol., xii, 780.
WYMAN, L. C. 1928a. This Journal, lxxxvi, 528.
1928b. This Journal, lxxxvii, 42.
WYMAN, L. C. AND C. TUM SUDEN. 1929. This Journal, lxxxix, 362.

THE ALLEGED INTERRELATIONSHIP OF THE ADRENAL CORTICAL HORMONE AND THE GONADS

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Indirect evidence accumulated over a number of years has given support to the idea that a functional interrelationship exists between the adrenal cortex and the gonads. The most persuading evidence of such a relationship has been that of a clinical nature. Something over 50 cases have been reported in which tumors of the adrenal cortex have been associated with pubertas precox, virilism, hirsutimus and pseudohermaphroditism (Healy and Guy, 1931; Glynn, 1911). With experimental work not definite, apparently the strength of this clinical evidence has been sufficient to convince many physiologists of an important adrenal-gonad relationship; and this idea is customarily stated in text-books of physiology.

The subject has not been neglected from an experimental standpoint. Some have reported adrenal hypertrophy during pregnancy and lactation. This was not found by Donaldson (1924), who reviews the literature. Altenburger (1924) and others have found adrenal hypertrophy after castration. Also, numerous studies have been made of sexual functions and changes as a result of experimentally-produced adrenal insufficiency, with varying results (Wyman, 1928; Shiffer and Nice, 1930; Del Castillo, 1928). One criticism can be applied to this work. The physiological upsets that result from total adrenal ablations are so extensive and profound that an effect on the gonads may well be altogether secondary to this general metabolic disturbance, and not directly caused by a lack of the cortical hormone. Martin (1928) offered evidence to show that any oestrous upset in adrenalectomized rats was of hypophyseal origin. Extensive degenerative changes in the testicular tubules of adrenalectomized rats were reported by Freed, Brownfield and Evans (1932). This was not verified by Gaunt (1932).

The most satisfactory approach to an experimental analysis of the problem is to use normal animals, and in one way or another overdose them with the cortical hormone. Thus, the complicating factors that can obscure results in adrenal insufficiency are avoided. This approach to the problem was made by Corey and Britton (1931). They reported the induction of a

precocious sexual maturity by repeated injections of a cortical extract prepared by them after the method of Swingle and Pfiffner (1930, 1931). The effects they obtained were not striking in the sense of those obtained by pituitary injections, and they could be obtained only after relatively long injections of large amounts of the extract. They described both a precocious ovulation and appearance of sperm.

Connor (1931) denied these conclusions and reported that he could induce no sexual precocity by a Swingle-Pfiffner preparation made by himself, while a saline extract of the adrenal cortex would cause a cessation of oestrus in mature animals.

The purpose of the present investigation was to repeat, and in some ways extend, part of the earlier work on the overdosing of normal animals with the cortical hormone, inasmuch as no agreement had been reached between previous workers. We used an extract of assayed potency, and if any effect had been obtained, it could have been stated in actual terms of the unit amount of hormone required.

Young rats and young chickens, prior to the age of sexual maturity, were used. The rats were injected intraperitoneally, and the injections given in divided doses twice daily. The chicks were injected subcutaneously once daily. Litter mate controls were used in the work on rats, and control chicks were of the same hatching as the experimental animals. Control animals were injected with normal saline solution in amounts equal to the volume of extract injected.

The extract used was prepared in the laboratories of Parke, Davis & Co., under the trade name of *Eschatin*, and is made from whole adrenal glands. It was part of the supply furnished by them to Drs. W. W. Swingle and J. J. Pfiffner for testing. The particular lot used assayed 20 dog units[1] per cubic centimeter—the assay being made in the research laboratories of Parke, Davis & Co., and checked in Professor Swingle's laboratories at Princeton University. The extract assayed 1 to 500,000 parts adrenalin—this adrenalin assay being made in the Parke, Davis & Co. laboratories.

Rat litters ranging from 12 to 16 days of age at the time of starting injections were used. Injections were stopped, and animals killed for autopsy, at 20, 30, 41, 49 and 51 days of age—after periods of injection ranging from 16, 24, 27, 32 and 37 days. Amounts of extract representing 5, 10 and 20 dog units (0.25, 0.5 and 1 cc.) per day for the entire injection-period were tried. These dosages were those which would maintain an adrenal-

[1] "A dog unit is defined as the minimum daily kilogram dose of cortical hormone necessary to maintain normal physiological conditions in the adrenalectomized dog for a period of 7 to 10 days; the two criteria of normal physiological condition being maintenance of body weight and blood level of non-protein-nitrogen (or urea)." (Harrop, Pfiffner, Weinstein and Swingle, 1932.) This method of assay has been very satisfactory in standardizing extracts.

ectomized dog of from 5 to 20 kgm. Previous work of this type has not been done on materials of measured potency.

It was thought that possibly in the chicken, a form in which the comb growth offers a conveniently measurable index of gonad activity, and also a form in which drastic modification or even reversals of sex appear in nature or can be produced experimentally, a symptomatology more nearly simulating the cases of adrenal tumors might be obtained. On the chickens, weight records and comb measurements were taken on alternate days.

Two groups of chickens were used. In one group, injections of 10 dog units (0.5 cc.) per day were begun at 6 days of age, and the animals were autopsied at 54 days of age after an injection period of 48 days. In the other group, injections of 10 dog units per day were started at 28 days of age, and animals autopsied after injection periods of 11, 20 and 32 days.

Fifteen chickens and 18 rats were extract treated.

At the end of the experiment, the animals were killed for autopsy, the reproductive tracts and other tissues dissected out. Tissues were then fixed in Bouin's fluid, cleared in xylol, sectioned and stained in hematoxylin and eosin. In the female rats, the reproductive tracts were photographed immediately after dissection to indicate relative sizes of control and experimental animals.

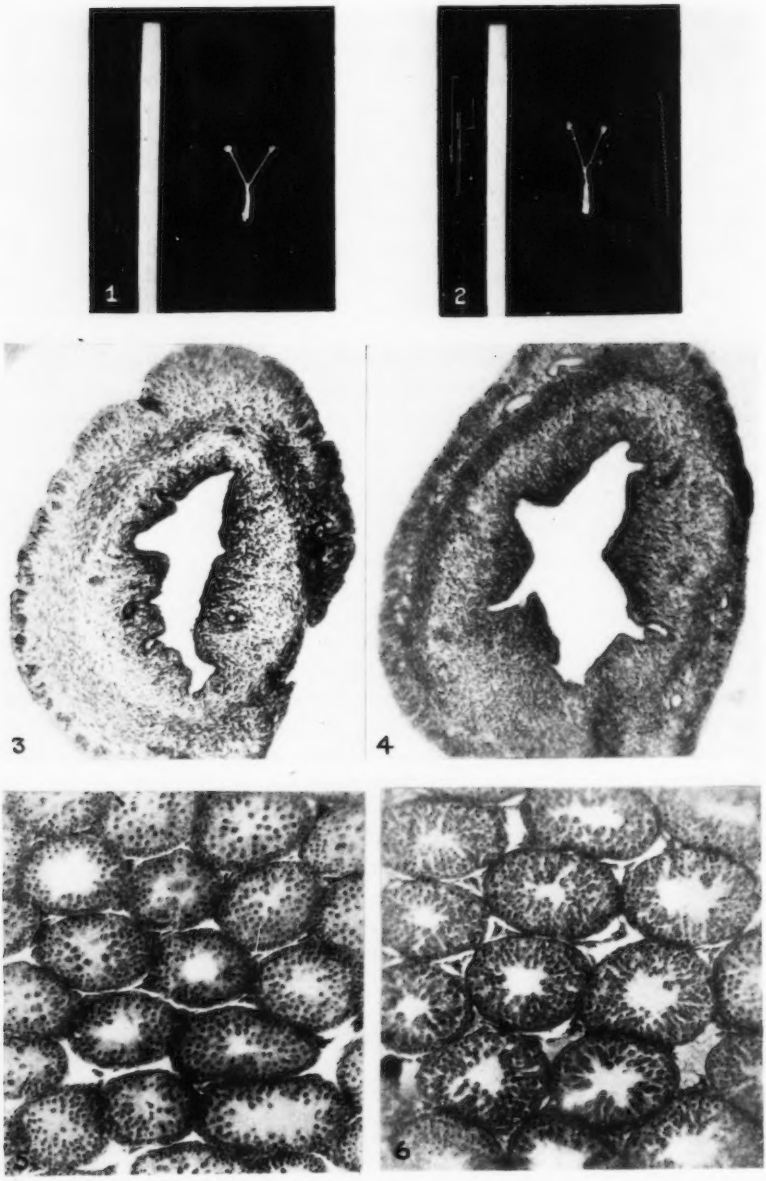
Results. The results were entirely negative.[2] No indication of precocious sexual maturity or of other effects of the prolonged treatment were discerned either in the rats or chickens. During the course of treatment of the rats, opening of the vagina was noted in only two cases. Although both of these cases were treated animals, the vaginal opening was well within expected normal limits, occurring at 50 and 49 days of age. Treatment was carried on well beyond the limits when in most cases either ovulation or the appearance of spermatozoa would have been of any significance. This was done to see if any effects of the prolonged injections could be observed. In no animal did ovulation occur, or were corpora lutea present. And no macro- or microscopic changes appeared in the uterus (figs. 1, 2, 3 and 4).

Neither in rats or the chickens did sperm occur in the testes, nor were there other characteristics that indicated any advanced maturity of treated animals (fig. 5, 6).

Gross body weight was not affected.

Discussion. The idea of an adreno-gonadal interrelationship carries with it the weight of the conviction of many experimental and clinical workers. Yet the case has never been fully demonstrated, and few experi-

[2] The results found here on young rats are in accord with the unpublished observations of Dr. H. O. Haterius of N. Y. University, who did similar experiments with some of the early and less potent Swingle-Pfiffner cortical preparations. We repeated the work to see if the better present-day extracts might be effective.



Figs. 1–6

mental findings pointing to that end have gone unchallenged. On theoretical grounds it could be argued that the extraction procedure of removing from adrenal tissue the life-maintaining hormone may separate out or destroy a sex principle. And it is the opinion of Corey and Britton that a distinct sex hormone exists in their extracts. However, adrenalectomized male and female dogs, kept alive by Doctors Swingle and Pfiffner for 14 months by their most highly purified cortical preparations, breed, become pregnant, deliver and lactate normally (in press). Certainly nothing essential to reproductive processes is lacking when the life-maintaining hormone is administered. These facts lead us to doubt the validity of the assumption of a direct interrelationship between the adrenals and gonads under normal conditions. How to correlate this conclusion with the clinical evidence drawn from observations of pathological conditions, is not clear. That other factors are involved in these pathological cases besides upsets in the adrenals and gonads alone is probable.

SUMMARY

Immature rats and chickens were treated over long periods with doses of cortical hormone varying from 5 to 20 dog units per day. This treatment failed to produce any effects on the reproductive system.

The authors wish to express their thanks to Dr. W. W. Swingle, in whose summer laboratory at The Biological Laboratory, Cold Spring Harbor, this work was carried out, for his coöperation and advice; and to Mr. J. H. Birnie for generous assistance with the photography.

BIBLIOGRAPHY

ALTENBURGER, H. 1924. Pflüger's Arch., ccii, 168.
CONNOR, C. L. 1931. Proc. Soc. Exp. Biol. and Med., xxix, 131.
COREY, E. L. AND S. W. BRITTON. 1931. This Journal, xcix, 33.
DEL CASTILLO, E. B. 1928. Compt. Rend., xcix, 1403.

Fig. 1. Shows reproductive tract of female rat of 41 days age that had been treated for 30 days with 10 dog units of *Eschatin* per day (rat C-5).

Fig. 2. Shows reproductive tract of litter mate control of above, which had been treated for same period with 0.5 cc. normal saline (rat C-4).

Fig. 3. Shows cross section of uterus of rat C-4. Whole reproductive tract shown in figure 2.

Fig. 4. Shows cross section of uterus of rat C-5. Whole reproductive tract shown in figure 1.

Fig. 5. Shows cross section of testis of rat 51 days of age that had been treated with 10 dog units of *Eschatin* per day for 40 days (rat E-7).

Fig. 6. Shows cross section of testis of litter-mate control that had been injected with 0.5 cc. daily of normal saline. In this case the control testis was nearer maturity than that of the experimental animal. This difference was apparantly only that of normal variation.

DONALDSON, J. C. 1924. This Journal lxviii, 517.
FREED, S. C., B. BROWNFIELD AND H. M. EVANS. 1931. Proc. Soc. Exp. Biol. and Med., xxix, 1.
GAUNT, R. 1933. This Journal, ciii, 494.
GLYNN, E. E. 1911. Quart. Journ. Med., v, 157.
HARROP, G. A., J. J. PFIFFNER, A. WEINSTEIN AND W. W. SWINGLE. 1932. Proc. Soc. Exp. Biol. and Med., xxix, 449.
HEALY, C. E. AND C. C. GUY. 1931. Arch. Path., xii, 543.
MARTIN, S. J. 1932. This Journal, c, 180.
PFIFFNER, J. J. AND W. W. SWINGLE. 1931. This Journal, xcvi, 180.
SCHIFFER, A. L. AND L. B. NICE. 1930. This Journal, xcv, 292.
SWINGLE, W. W. AND J. J. PFIFFNER. 1930. Sci., lxxi, 321.
WYMAN, L. C. 1928. This Journal, lxxxvi, 528.